



# **Granular biomasses for biological treatment of breeding wastewater**

## **Dissertation**

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<sup>\*)</sup> Either the German or the Italian form of the title may be used.



*Ai miei genitori*





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## LIST OF SYMBOLS AND ABBREVIATIONS

AGS	AEROBIC GRANULAR SLUDGE
ANAMMOX	ANAEROBIC AMMONIUM OXIDIZING BACTERIA
AOB	AMMONIUM OXIDIZING BACTERIA
ATU	ALLYLTHIOUREA
BC1	CYTOCHROME BC1 COMPLEX
BNR	BIOLOGICAL NUTRIENT REMOVAL
$C_{SI}$	OXYGEN CONCENTRATION IN THE BIOFILM INTERPHASE
$C_{XF}$	BIOMASS IN THE BIOFILM
CANON	COMPLETELY AUTOTROPHIC NITROGEN REMOVAL OVER NITRITE
CEC	CONTAMINANTS OF EMERGING CONCERN
COD	CHEMICAL OXYGEN DEMAND
CSTR	CONTINUOUS STIRRED TANK REACTOR
CYT	CYTOCHROME C
C/N	CARBON TO NITROGEN
C-REMOVAL	REMOVAL OF ORGANIC CARBON
D	OXYGEN DIFFUSION COEFFICIENT
DGAO	DENITRIFYING GLYCOGEN ACCUMULATING ORGANISMS
DPAO	DENITRIFYING POLY-PHOSPHATE ACCUMULATING ORGANISMS
DO	DISSOLVED OXYGEN
EBPR	ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL
EGSB	EXTENDED GRANULAR SLUDGE BED
EPS	EXTRACELLULAR POLYMERIC SUBSTANCES
ETSS	EFFLUENT TOTAL SUSPENDED SOLID
GAO	GLYCOGEN ACCUMULATING ORGANISMS
HEPES	N-2-HYDROXYETHYL-PIPERAZINE-N'-2-ETHANE SULFONIC ACID
HRT	HYDRAULIC RETENTION TIME
HZS	HYDRAZINE SYNTHASE
H/D	HEIGHT TO DIAMETER
$K(T)$	CONVERSION RATE AT TEMPERATURE
MLSS	MIXED LIQUOR SUSPENDED SOLIDS
MLVSS	MIXED LIQUOR VOLATILE SUSPENDED SOLIDS
NIRS	NITRITE REDUCTASE
NOB	NITRITE OXIDIZING BACTERIA
N-REMOVAL	REMOVAL OF NITROGEN COMPOUNDS
OLAND	OXYGEN-LIMITED AUTOTROPHIC NITRIFICATION-DENITRIFICATION
OLR	ORGANIC LOADING RATE
P	OVERPRESSURE
PAO	POLY-PHOSPHATE ACCUMULATING ORGANISMS
PHA	POLYHYDROXYALKANOATES
PHB	POLYHYDROXYBUTYRATE
PN	PROTEIN
PS	POLYSACCHARIDE

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POLY-P	POLY-PHOSPHATE
P-REMOVAL	PHOSPHATE REMOVAL
Q	UBIQUINONE
QW	WASTE FLOW RATE
$Q_{O_2}^{MAX}$	MAXIMUM SUBSTRATE UPTAKE RATE
R	IDEAL GAS COEFFICIENT
RAS	RETURNED ACTIVATED SLUDGE
SAA	SPECIFIC ANAMMOX ACTIVITY
SAUR	SPECIFIC AMMONIUM UPTAKE RATE
SBR	SEQUENCING BATCH REACTOR
SGBR	STATIC GRANULAR BED
SHARON	SINGLE REACTOR SYSTEM HIGH AMMONIUM REMOVAL OVER NITRITE
SND	SIMULTANEOUS NITRIFICATION-DENITRIFICATION
SNDP	SIMULTANEOUS NITRIFICATION AND DENITRIFICATION AND PHOSPHORUS REMOVAL
SPUR	SPECIFIC PHOSPHATE UPTAKE RATE
SRT	SOLIDS RETENTION TIME
SVI	SLUDGE VOLUME INDEX
T	TEMPERATURE
TN	TOTAL NITROGEN
TP	TOTAL PHOSPHORUS
TSS	TOTAL SUSPENDED SOLID
UASB	UP-FLOW ANAEROBIC SLUDGE BLANKET
VER	VOLUME EXCHANGE RATIO
VFA	VOLATILE FATTY ACIDS
$V_{HS}$	VOLUME OF THE HEAD SPACE
WAS	WASTE ACTIVATED SLUDGE
WWTP	WASTEWATER TREATMENT PLANT
X	BIOMASS CONCENTRATION
$\delta_{PF}$	OXYGEN PENETRATION DEPTH



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## **ABSTRACT**

Over the last few years, biogranulation has been regarded with increasing interest according to granules advantages over conventional activated sludge. This thesis focused on two specific granular consortia: the anammox bacteria, as a specific case of denitrifying anaerobic granules, and the aerobic granular sludge. Specifically, both solutions were evaluated for swine wastewater treatment. As swine wastewaters contain high concentrations of veterinary antibiotics, the potential negative effects of these compounds on microbial activities need to be studied carefully. In detail, the research activity focused on the evaluation of the most advantageous operating conditions for aerobic granules cultivation, and on the estimation of the toxicity of three veterinary antibiotics respectively on anammox granules, aerobic granules and conventional activated sludge. The short- and long-term toxicity of selected compounds on the anammox biomass was evaluated through batch tests designed to estimate the Specific Anammox Activity. Moreover, the short-term toxicity of combined concentrations of antibiotics was evaluated so to verify whether a synergistic effect could establish. Results demonstrated that at antibiotics concentrations expected for swine wastewaters, the application of the anammox process seems conceivable, as at those levels, the activity was just slightly reduced. Aerobic granulation of activated sludge was achieved in a Sequencing Batch Reactor fed with a mixture of synthetic medium and domestic sewage. The operating parameters, which were found to influence mostly granules stability and performances were the selection of slow, growing organisms, dissolved oxygen concentration, feeding strategy and temperature. The inhibitory effect of target compounds on aerobic granules was then assessed for ammonium oxidizing bacteria and phosphorus accumulating organisms, which demonstrated to be highly resistant to those compounds. Finally antibiotics toxicity was assessed for granular and flocculent microbial populations performing the same nitrifying and denitrifying activities. Results highlighted the higher resistance of the microbial guilds performing aerobic ammonium oxidation present in granules towards all the tested antibiotics compared to conventional activated sludge. Anammox bacteria susceptibility was instead depending on the specific antibiotic tested, leading to an overall inhibition nearly comparable to that of conventional flocculent denitrifiers.

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# SUMMARY

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## Granular process: a sustainable alternative to activated sludge

The activated sludge process is actually the most widespread biotechnology for wastewater treatment; however, particularly when applied to biological nutrient removal (BNR), the operation of the process often exhibits some operational dysfunctions such as: high tendency to bulk, need of solid-liquid separation, significant mass of waste activated sludge produced and low volumetric removal rates.

Therefore, biogranulation process has been regarded with increasing interest over the last few years according to granules advantages over conventional activated sludge. In fact, granules are denser and more compact than flocs, thus showing excellent settling properties and allowing high biomass retention in the reactor. On the one hand, the great settleability leads to a long solid retention time (SRT), which results in decreased sludge production; on the other hand, high biomass concentration significantly increases the volumetric load conversion rates, the capacity to withstand high organic loading rates and leads to a space requirement reduction due to the use of compact reactors.

This study focused on two specific granular consortia that recently raised researchers' attention: the anammox biomass (ANAerobic AMMonium OXidizing bacteria), as a specific case of anaerobic granules, and the aerobic granular sludge (AGS). According to their microbial composition and related metabolism, these granular technologies have been chosen in this study as innovative process for biological treatment of swine wastewaters.

Anammox bacteria are, in fact, capable of autotrophic ammonium oxidation with nitrite as electron acceptor and are considered one of the most innovative and sustainable biological nitrogen removal alternatives to traditional nitrification-denitrification technology. Starting from 1999, when the molecular identification of the bacterium responsible for this reaction was achieved, this technology was successfully implemented in full-scale wastewater treatment systems. To date, anammox process is mainly applied for ammonium removal from sludge digester rejection water and for the treatment of effluent from different industrial (food industry, fertilizer industry, petrochemical industry, metal and mining industry) wastewaters, characterized by a high ammonium concentration ( $\text{NH}_4^+\text{-N} > 100 \text{ mg L}^{-1}$ ), low carbon to nitrogen (C/N) ratio and temperatures exceeding 25°C.

Aerobic granular sludge technology was instead developed in the last two decades as a technology capable of simultaneous C, N and P removal, thanks to the peculiar characteristics of the granule, which presents aerobic, anoxic and anaerobic zones where heterotrophs, nitrifiers, denitrifiers, phosphorous and glycogen accumulating organisms (PAO/GAO) can proliferate. Although aerobic granules have to date been cultivated either at laboratory, pilot

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and full-scale using synthetic, domestic and industrial wastewaters, under a wide range of loading rates (from 0.7 to 15 Kg COD m<sup>-3</sup> d<sup>-1</sup>), this process still presents some bottlenecks, mostly related to the long-term granules instability, and a further optimization is then needed. Compared to conventional manure treatment scheme, the application of a combined partial nitrification-anammox process instead of common nitrification-denitrification process for the treatment of the digester supernatant, can reduced by 57% the oxygen demand and by 100% the costs associated to additional external carbon dosage. Moreover, the low anammox yield leads to a very small sludge production. Taking into account the mainstream manure treatment, 75% less area and 30% less energy consumption can be achieved by applying AGS instead of conventional AS. In fact, thanks to the great settleability of aerobic granules, no secondary clarifiers are needed, while simultaneous C, N and P removal cuts out recycling costs. Finally, the selection for slow-growing organisms leads to less aeration requirements and to a reduced sludge production.

However, swine wastewater also contains high concentrations of veterinary antibiotics, As antibiotics directly inhibit biological activities, they are likely to exert an inhibitory effect on the biodegradation process commonly applied for nutrient removal, thus negatively affecting the efficiency of the treatment processes. So, in order to apply successfully granular technology for the treatment of pig slurry, the potential negative effects of the veterinary antibiotics present in these wastewaters need to be studied.

## **Specific objectives of the research**

This study is part of two different research projects: “PRIN-Emerging contaminants in air, soil, and water: from source to the marine environment”, financed with Decree of the Italian Ministry of Education, Universities, and Research (MIUR), and “CARBALA—CARbon BALancing for nutrient control in wastewater treatment”, a Marie Curie Action within the International Research Staff Exchange Scheme (IRSES), financed by the European Union.

The research activity has been conducted partly in Florence and partly at the environmental engineering laboratory of the Department of Civil Engineering of the University of Manitoba, Winnipeg, Canada. Specifically, preliminary studies regarding the aerobic granulation process were conducted at the environmental engineering laboratory of the University of Manitoba, while the start-up of an aerobic granular Sequencing Batch Reactor and the inhibitory tests on aerobic and anaerobic (anammox) granular sludge were entirely managed within the Department of Civil and Environmental Engineering-DICEA of the University of Florence.

The objectives of this study were:

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1. Evaluation of the most advantageous operating conditions for aerobic granules cultivation, aimed at translating this biological process into an economically feasible full-scale system;
  2. Assessment of the inhibitory effect of veterinary antibiotics on the anammox biomass and comparison with the inhibition caused on conventional activated sludge;
  3. Assessment of the inhibitory effect of veterinary antibiotics on aerobic granular biomass and comparison with the inhibition caused on conventional activated sludge;
  4. Evaluation of the overall advantages of granular biomass systems over conventional activated sludge process based on results achieved from point 2 and 3.

## Contents

The research project activities are presented in the following chapters as summarized below:

CHAPTER 3 describes the evaluation of the short- and long-term inhibitory effect on the anammox process of three veterinary antibiotics (tiamulin, doxycycline and enrofloxacin) commonly administered to Italian livestock, and consequently present in the digester supernatant. Since the cultivation of anammox granules has been extensively studied, the enriched biomass used to assess the inhibitory effect of the selected antibiotics, originated directly from the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam, The Netherlands.

CHAPTER 4 focuses on the aerobic granules cultivation and includes two sections. The first section describes a preliminary study regarding aerobic granulation process of activated sludge at laboratory-scale and all the related bottlenecks. The second section describes the start-up of an aerobic granular Sequencing Batch Reactor for the purpose of achieving the *inoculum* for the subsequent inhibitory tests.

CHAPTER 5 describes the evaluation of the short-term inhibitory effect of selected antibiotics on two microbial populations present within aerobic granules and responsible for the nitrogen and phosphorus conversion bioprocesses.

CHAPTER 6 focuses on the comparison between the inhibitory effect of selected compounds on the nitrogen conversion bioprocesses performed by granular and conventional suspended biomass. Specifically four microbial populations were studied: ammonium oxidizers developed within aerobic granules, ammonium oxidizers present in conventional activated sludge flocs, granular autotrophic denitrifiers (anammox) and heterotrophic denitrifiers developed within activated sludge flocs.

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# **CHAPTER 1**

# **GRANULAR SLUDGE**

# **PROCESSES**

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## Conventional biological wastewaters treatment

The main aim of biological wastewaters treatment is the removal of organic compounds and nutrients, which may be present inside domestic or industrial effluents, and may be harmful for the environment when discharged without being previously treated.

The activated sludge process is nowadays one of the most widespread biological technology for wastewaters treatment. In this system, a mixed culture of suspended microorganisms (bacteria, fungi, protozoa and algae) ensures the organic carbon and nutrient removal from the influent and leads to a high quality effluent. Activated sludge system consists of three basic components: 1) an aeration tank in which the microorganisms are kept in suspension and biochemical processes take place, 2) a secondary clarifier, in which a separation of the activated sludge from the treated waste takes place by gravity settling, 3) a sludge recycling system for returning part of the activated sludge (returned activated sludge, RAS) back to the beginning of the process in order to maintain the desired concentration of organisms in the basin, while the other part is withdrawn from the system and then directed to the sludge treatment stage (waste activated sludge, WAS) (Fig. 1.1).

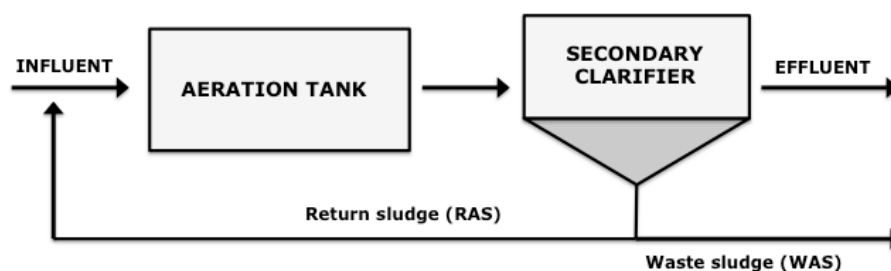


Figure 1-1. Conventional activated sludge process scheme.

Carbon and nutrient removal is possible because of the several different microbial groups which explicate their biochemical processes in separated stages of the reaction tank: in aerobic tanks oxidation of organic carbon (heterotrophs) and ammonia (nitrifiers) is carried out, while in anoxic tanks and anaerobic zones denitrification (denitrifiers) and phosphate removal (phosphorus accumulating bacteria, PAO) respectively take place.

### 1.1.1 Organic carbon removal

Typically polysaccharides, lipids and proteins are the most common high molecular weight compounds present in a typical municipal wastewater. These compounds need to be hydrolysed before they can be used by the biomass. After the extracellular hydrolysis of



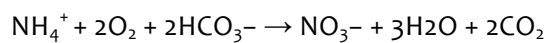
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organic polymers, the single molecules are transferred to the cells where they are oxidized to CO<sub>2</sub> using oxygen (heterotrophs) or nitrite/nitrate (denitrifiers) as electron acceptor, and part of the organic carbon is assimilated to new biomass.

### 1.1.2 Nitrogen removal

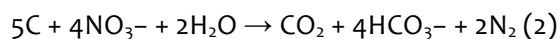
Nitrogen entering a wastewater treatment plant (WWTP), mostly in the form of ammonium, can be biologically removed. Conventionally nitrogen removal is achieved through autotrophic nitrification and heterotrophic denitrification.

Nitrification is the biological process where ammonium is oxidized to nitrate with oxygen as electron acceptor:



This is a two-step pathway: first the oxidation of ammonium to nitrite is carried out by ammonium-oxidizing bacteria (AOB), such as *Nitrosomonas* or *Nitrosospira*, and then the produced nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB), such as *Nitrobacter*.

During the subsequent denitrification, nitrate or nitrite is used as electron acceptor for the oxidation of organic carbon and reduced to dinitrogen gas under anoxic conditions:



Simultaneous nitrification/denitrification process (SND) can also take place when specific conditions occur: oxygen penetration depth within the flocs must be limited, and the organic substrate must be present in the inner anoxic zone for denitrification.

### 1.1.3 Phosphorus removal

Phosphorus entering a WWTP is commonly in form of orthophosphate and it results from the degradation of organic molecules and from the hydrolysis of polyphosphates used in detergents. In conventional activated sludge plants, phosphorus is removed through combined biological removal and chemical precipitation systems (using calcium, aluminium or iron salt addition) and the percentage biologically removed is utilized only for the synthesis of new biomass. The enhanced biological phosphorus removal (EBPR) can be achieved through an enrichment of the PAO culture. This enrichment is possible by recirculating the sludge through an anaerobic phase, where volatile fatty acids (VFA) are abundant, and then through an aerobic/anoxic one. According to PAO metabolism, during the anaerobic phase VFA are

stored intracellularly as polyhydroxyalkanoates (PHA). The energy required for the transport of VFA through the cell membrane, and their internal storage, originates by the hydrolysis of the intracellular polyphosphate (poly-P) chains to orthophosphate, which is released into the bulk. The reducing power necessary for the conversion of acetate to PHB is provided by glycogen hydrolysis. Moreover, the anaerobic hydrolysis of poly-P also provides the energy required for the anaerobic maintenance. During the aerobic (or anoxic) phase, PAO utilize the PHA internally stored for cell growth, polyphosphate synthesis and glycogen restore (Fig. 1.2). The orthophosphate uptake under aerobic conditions is higher than its release in the anaerobic stage because of the cell growth. Net P-removal from the wastewater is then achieved through the removal of waste activated sludge (WAS), rich in poly-P content, from the system.

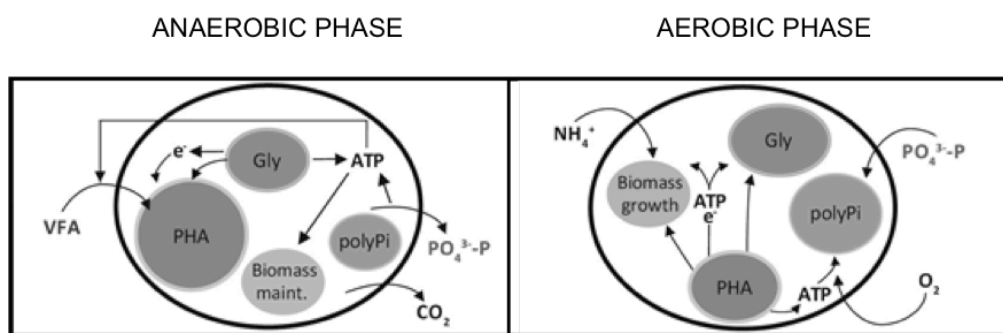


Figure 1-2. Anaerobic and aerobic metabolism of phosphate accumulating organisms.

The efficiency of a biological wastewater treatment system is highly related to some factors such as the active biomass concentration, overall biodegradation rate, reactor configuration, operating conditions and influent characteristics.

Despite the common use of activated sludge system, this technology exhibits some operational dysfunctions and instabilities and the main disadvantages can be summarized as follow:

- High tendency to bulk;
- Solid-liquid separation after treatment, which requires secondary clarifiers with high costs associated to their building and maintenance and a strict correlation between sludge retention time (SRT) and sludge settleability;
- Significant volume of sludge production, which consequently has to be treated.

Moreover, biomass characterized by low settling properties involves additional wastewater treatment costs emerging from chemical dosing and process modification, as well as higher

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nutrient loads in the treated effluent from increased suspended solids [1,2]. The settling properties of the sludge and, subsequently, the solid-liquid separation efficiency can be improved by converting the floccular activated sludge into a denser and more compact consortium like biofilms or granular sludge. By additionally eliminating secondary clarifiers and providing simultaneous nitrogen and phosphorus removal in an aerated sequencing batch reactor (SBR) system, granular sludge can decrease the required plant footprint and energy consumption by 75% and 20% respectively [3].

## **Granular sludge**

Over the last decades, granulation has been regarded with increasing interest according to its efficiency in biological wastewater treatment [4]. Granules are characterized by a greater density, more compact structure and larger diameter compared to bioflocs ( $>200\ \mu\text{m}$ ) [5]. Since granular sludge is constituted by compact and dense microbial aggregates, it allows a greater biomass concentration and longer SRT in the reactor. Higher biomass concentration significantly increases the volumetric load conversion rates of microorganisms in the aggregates and a longer SRT results in decreased sludge production [6]. The robust microbial structure of granular sludge increases the ability to withstand high organic loading rates, while the presence of an extracellular matrix of polymeric substances (EPS) ensures a better resistance to toxic pollutants. Biogranulation requires cell-to-cell interactions and involves biological, physical and chemical phenomena. Granules are considered as a special case of biofilm, containing millions of different microbial cells associated to create a compact and spherical shape [7].

Granular sludge was first described in 1980 for anaerobic systems [8], while aerobic granulation was first reported in 1990's [9,10]. Anaerobic granulation has been extensively studied and applied in Up-flow Anaerobic Sludge Blanket (UASB), Extended Granular Sludge Bed (EGSB) and Static Granular Bed (SGBR) reactors [11]; the efficiency and practicability of UASB reactors in removing biodegradable organic matter from municipal and industrial wastewater have been successfully proven [9,12]. However, anaerobic granulation technology exhibited several drawbacks requiring a long start-up period, high operating temperature and high organic strength wastewater [13]. Thus, the functional flexibility of aerobic granular sludge technology towards changes in operating conditions (temperature and organic loading rate) and its possible application to biological nutrient removal arose, in the last two decades, research interests.

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#### 1.1.4 Mechanisms of granulation

Biogranulation process can be defined as the aggregation of cells to form a stable, contiguous, and multicellular consortium [14]. According to Fitzpatrick et al. [15] the possible advantages for bacteria to aggregate are:

- Development of heterogeneous populations of syntrophic microorganisms;
- Promotion of interactions and genetic exchange between organisms;
- Protection from predators (e.g., ciliates);
- Establishment of suitable microenvironments within the granule, despite disadvantageous growing conditions in the bulk solution.

Granulation is a gradual process usually initiating from fluffy seed sludge to compact aggregates, then to granular sludge and finally to mature granules, without using external carriers [16].

Liu and Tay [13] suggested a general four-phase model for granulation process:

1. In the first phase cell movement initiates bacterium-to-bacterium contact or bacterial attachment onto an external carrier which acts as a core;
2. In the second phase stabilization of attractive forces and immobilization of different cells take place;
3. In the third phase granulation occurs and cell aggregation transforms into a highly organized microbial structure;
4. In the fourth phase maturation of the three-dimensional aggregates, in terms of granules stability and compactness, is conducted by hydrodynamic shear force.

The interaction between bacteria includes repulsive electrostatic forces, attractive van der Waals forces and hydration interactions. To achieve cells aggregation many conditions have to be satisfied in order to promote microbial adhesion [17]. Some of these conditions are related to the characteristics of the involved microorganisms, such as cell surface hydrophobicity [18], bacterial physiology (e.g., the production of extracellular polymers) and bacterial morphology.

#### 1.1.5 Characteristics of granular sludge

Compared to activated sludge flocs, granular biomass exhibits the following characteristics [13, 19]:

- Regular, smooth, round shape;

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- Excellent settleability;
  - Dense and strong microbial structure;
  - High biomass retention;
  - Ability to withstand high organic loading rates;
  - Tolerance to toxic compounds.

A large number of parameters were studied to investigate granules characteristics, including physical (settling velocity, density, specific gravity and sludge volume index), chemical (cell surface hydrophobicity and extracellular polymeric substances) and biological (microbial composition) factors.

#### **1.1.5.1 Physical characteristics**

##### *Shape and size*

Granules are typically spherical or ellipsoidal with a smooth outer surface. The granule's morphology can be affected by many factors, such as organic loading rate, type of applied substrate, microbial population and operational conditions. The average size of granules in aerobic processes is the result of two opposing phenomena: (1) biomass growth; and (2) cell detachment due to hydrodynamic shear forces. [20] It has been shown that the average diameter of both anaerobic and aerobic granules varies from 0.2 to 5.0 mm and is greater than the average diameter of activated sludge flocs. [4, 17, 21].

##### *Settleability*

The settleability of activated sludge is directly related to the biomass concentration and solid-liquid separation efficiency. The settling velocity of granular sludge can vary from 25 to 70 m h<sup>-1</sup> and is significantly higher than activated sludge flocs (i.e., 7 to 10 m h<sup>-1</sup>) [22]. Higher settling velocities allow for greater biomass concentrations that consequently enhance removal capacity by increasing the active biomass and maintaining slow-growing bacteria [23]. The sludge volume index (SVI) of granular sludge is usually below 80 mL gTS<sup>-1</sup> and values as low as 20 mL gTS<sup>-1</sup> have been reported [24]. Low SVI values are due to the high density and compact structure of granules.

#### **1.1.5.2 Chemical characteristics**

##### *Hydrophobicity and surface charge*

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Adhesion is the result of interaction between attractive and repulsive forces among different approaching surfaces [25]. In general, microbial cells adopt a disassociated state, while aggregation is usually a response to stressful conditions.

Bacterial cell surface hydrophobicity represents one of the most important triggering factors for granulation. Cell surface hydrophobicity is usually associated with the presence of specific bacterial fibrils and cell wall proteins [26, 27]; there are also some polysaccharides (e.g., alginate) that are hydrophobic or include hydrophobic regions due to their molecular structure [28].

According to van Oss [29], in biological systems hydrophobic interactions are usually the strongest among all non-covalent interactions. From a thermodynamic point of view, an increase in the hydrophobicity of the cell surface causes a decrease in the Gibbs free energy of the system and promotes the adhesion between bacterial cells submerged in an aqueous solution [20]. The decrease in Gibbs free energy is a result of displaced ordered water molecules from the surfaces of interacting molecules to the bulk solution [30].

An inverse correlation between cell surface hydrophobicity and surface negative charge has been demonstrated. Higher surface charges result in stronger polar interactions between extracellular polymeric substances (EPS) and water molecules. Therefore, higher cell hydrophobicity corresponds with reduced cell surface charge and leads to weakened repulsive forces between microbial cells. The surface hydrophobicity and charge of granules affect the production, composition, and physical characteristics of EPS [31].

#### *Extracellular polymeric substances*

Geesey [32] defined the EPS as “extracellular polymeric substances of microbiological origin that participate in the formation of microbial aggregates”. These polymers have been found in both anaerobic and aerobic granules where they represent a very important factor during the first phases of microbial adhesion and in granule stability. Their basic role seems to be the formation of an extracellular matrix where cells are trapped.

The predominant macromolecules involved in the EPS structure are: polysaccharides, proteins, lipids, nucleic, humic and uronic acids [33]. In addition, non-polymeric substituents such as acetyl, succinyl, pyruvyl and inorganic groups also can be found in the EPS composition. Proteins can be associated with lipids (lipoproteins) or covalently bound to carbohydrates (glycoproteins) [34, 35].

EPS are distinctively bound to the cell surface (cell-bound EPS) or excreted in the growth medium (soluble free EPS). The final placement can be due to an active transport from the

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metabolic origin to the cell surface, adsorption from the surrounding medium or release from the cell lysis [36]. Polysaccharides are the only components synthesized extracellularly, while the others are synthesized inside the cytoplasm and then excreted outside the cell wall [37, 38]. The ability to secrete EPS is widespread among microorganisms because it serves functions such as resistance against flocculation, adherence to surfaces, promotion of microbial aggregation and protection against specific and non-specific host immunity.

Under standard cultivation conditions the EPS production is normally low; an increase in this production occurs when bacterial cells are exposed to external stresses, which can be divided into two main groups [39]:

- Environmental changes which can alter the microbial community, increasing or decreasing the amount of EPS-producing bacteria;
- Environmental changes which can modify the metabolic pathway of EPS production of the unchanged microbial community.

Some studies regarding the EPS composition, presented proteins as the predominant constituent [40-42]. It must however be pointed out how the amount and composition of synthesized EPS are strictly related to the microbial species involved, to their growth phase and physiology, to the applied operating conditions and to the feed composition [43,44]. Since proteins have a high content of negatively charged amino acids, and they appear to represent the higher fraction of EPS, it is reasonable to believe they are more involved than polysaccharides in electrostatic bonds with divalent cations. EPS production can enhance aerobic granulation process affecting cell surface hydrophobicity and electric charge. Proteins are usually hydrophobic while polysaccharides are usually hydrophilic [45]. According to Zhang et al. [46] higher surface hydrophobicity was associated with higher protein to polysaccharide ratios (PN/PS) and lower surface negative charge. Since EPS is heterogeneous, hydrophobic and hydrophilic groups can be simultaneously present. This has two main consequences: (1) the overall hydrophobicity of the EPS matrix is the result of the average between its hydrophobic and hydrophilic components (e.g., depending on the PN/PS ratio) [47]; and (2) the cell surface hydrophobicity is strictly dependent on the composition of EPS [31]. Granular sludge was found to be less negatively charged than activated sludge and it has been proposed that EPS can enhance microbial adhesion by decreasing the negative charge of cell surfaces through cross-links with divalent cations (e.g.,  $\text{Ca}^{2+}$ ) [48].

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## Anaerobic granules: the anammox process

Conventional anaerobic granular sludge was first described in 1980 and it has been extensively applied in UASB [8] and EGSB [49] reactors for the removal of biodegradable organic matter from domestic and industrial wastewaters by converting it into biogas. This process presents several advantages over conventional activated sludge, which can be summarized as follows:

- Capacity to withstand high loading rates, due to the high settling properties of granular sludge, which allow an uncoupling of the hydraulic retention time from the solid retention time, and the high specific methanogenic activities;
- Reduction in the reactor size and the required area for the treatment;
- Lower operational costs, due to the absence of aeration since mixing is provided through the up-flowing liquid and the produced biogas [50].

On the other hand, anaerobic granular treatment exhibits some drawbacks. These include the need for a long start-up period, resulting from the low growth rate of methanogenic organisms, a relatively high operation temperature ( $>20^{\circ}\text{C}$ ) and unsuitability for low-strength organic wastewater. In addition, anaerobic granulation technology is not suitable for the removal of nutrients (N and P) from wastewater. In conclusion, conventional anaerobic treatment can be successfully applied for the treatment of nutrient-free industrial wastewaters containing high COD concentrations, or for sewage treatment in tropical and subtropical climates.

More recently, another group of anaerobic granules-forming bacteria has been discovered. This group is particularly interesting, since their metabolism represents a new biochemical pathway in the nitrogen cycle. The members of this new bacterial group are named ANoxic AMMonium OXidizers, (Anammox) and shortly after their discovery, they were applied for inorganic nitrogen removal from wastewater as described in the following paragraph.

### 1.1.6 The Anammox process

Nitrogen entering a wastewater treatment plant (WWTP), mostly in the form of ammonium, can be biologically removed. Conventionally nitrogen removal is achieved through autotrophic nitrification and heterotrophic denitrification. The combination of these two processes ensures a high removal efficiency and stability, but it is associated with high-energy costs of aeration (nitrification step) and it also discloses some deficiencies when applied to the treatment of influents characterized by a low C/N ratio, which require an external carbon



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dosage to support denitrification process.

Although the ammonium oxidation has been mainly investigated in aerobic conditions, theoretically it could be also used as an inorganic electron donor since the free energy associated with this reaction ( $\Delta G_o = -358 \text{ kJ mol}^{-1}$ ) is nearly as favourable as for the aerobic nitrification process [51]. The first evidence that this process could take place was in the early 1990s in a pilot-scale denitrifying reactor at the baker's yeast factory Gist-Brocades in Delft, The Netherlands [52]. Many attempts to isolate the organism responsible for this process followed this discovery, and in 1999, scientists succeeded in the molecular identification of the bacterium responsible for this reaction [53]. These bacteria were named anammox and are members of the order of Planctomycetales. They were found both in aquatic environments characterized by oxygen minimum zones, where they contribute significantly to  $\text{N}_2$  production, and in a wide range of terrestrial environments. To date, five different genera have been described: "*Candidatus Brocadia*" [53, 54], "*Candidatus Kuenenia*" [55], "*Candidatus Scalindua*" [56, 57], "*Candidatus Anammoxoglobus*" [58], and "*Candidatus Jettenia*" [59]. The "*Candidatus*" status is due to the fact that none of these genera have been isolated in pure culture. The divergence between the five genera is relatively large and the sequence identity on 16S rRNA gene level ranges from 87 to 99 % [60].

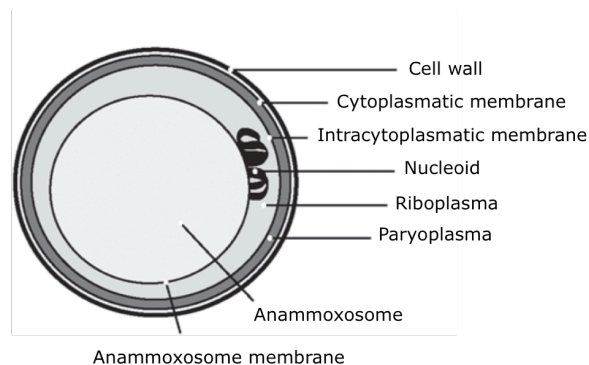
Since the main characteristic of these microorganisms is to oxidize ammonium to dinitrogen gas, with nitrite as electron acceptor under strictly anoxic conditions [53], they represent a cost-effective technology to remove ammonia and nitrite from industrial and domestic wastewaters.

### 1.1.7 Cell structure and physiology

Anammox bacteria are a recent discovered group of anaerobic granules-forming organisms. They are slow growing Gram-negative, characterized by coccoid cells associated together to form reddish-brown granules (probably due to cytochrome contents), with a doubling time of around 11 days. To date, no pure culture of these microorganisms has been isolated and only enriched cultures, which typically contain 60-80% anammox bacteria, exist.

Like other Gram-negative bacteria, anammox cell wall lacks in peptidoglycan [61], but differently from other Gram-negative it is not surrounded by any outer membrane. On the contrary, there are two membranes on the inner side of the cell wall: the cytoplasmic and the intracytoplasmic membrane (Fig. 1.3). The cytoplasmic compartment defined by these two membranes is named paryphoplasm, and its function has not been discovered yet. Proceeding towards the inner part of the cell, two other distinctive structures can be detected: the

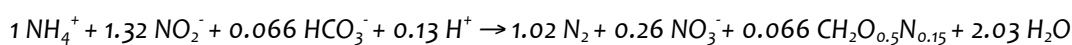
riboplasm, containing DNA, ribosomes and glycogen storages, and the anammoxosome. The anammoxosome occupies the 50–70% of the total cell volume and represents the energy production headquarter [62].



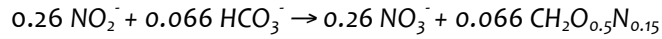
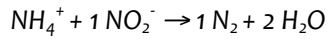
**Figure 1-3. Anammox cell structure (based on Jetten et al. [65]).**

The structure of all the intracellular membranes is a conventional single bilayer, but it is constituted by unique glycerolipids named ladderanes. Ladderanes structure includes glycerol moieties linked to fatty acid residues through both ester and ether bindings. The hydrocarbon tails comprise 3 or 5 linearly concatenated cyclobutane rings and this arrangement has so far been found only in anammox bacteria [63]. Moreover, ladderane lipids seem to be highly packed, thus reducing the permeability of the membrane and the loss of gaseous (nitric oxide) and toxic (hydrazine) intermediates.

Anammox bacteria are obligate anaerobes and their metabolism is reversibly inhibited at oxygen concentrations above 2  $\mu\text{M}$ ; moreover, anammox organisms have been reported to tolerate a pH range from 6.7 to 8.3 (with an optimum at 8.0) and a temperature range from 20°C to 43°C with an optimum at 35°C. Initially they have been described as strictly autotrophic bacteria, capable to oxidize ammonium to dinitrogen gas, with nitrite as electron acceptor, while recent studies demonstrated that some anammox bacteria (*Anammoxaglobus*, *Brocadia*, *Kuenenia*) may possess a mixotrophic metabolism, being able to use some organic compound as electron donors [58]. The energy for growth is achieved from the anaerobic conversion of ammonium and nitrite into  $\text{N}_2$ , while  $\text{CO}_2$  serves as the sole carbon source for the synthesis of cell biomass, according to the overall equation [64]:

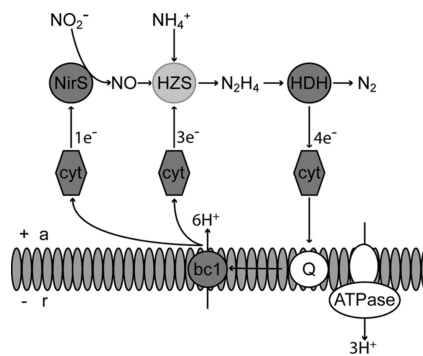


This reaction is the sum of two partial reactions, the first one related to the energy production process, and the other to carbon fixation into cell biomass:



According to the anammox stoichiometry this process allows over 50% of the oxygen to be saved, no organic carbon source is needed and a very low amount of sludge (0.11 g VSS/g  $\text{NH}_4\text{-N}$ ) is produced.

A model for the conversion of the substrates inside the anammoxosome is shown in Figure 1.4.



**Figure 1-4. Biochemical model of the anaerobic ammonium oxidation.**

This model considers three different reactions:

1. Reduction of nitrite to nitric oxide (NO) by nitrite reductase (NirS),
2. Condensation of nitric oxide and ammonium to hydrazine ( $\text{N}_2\text{H}_4$ ) by hydrazine synthase (HZS)
3. Oxidation of hydrazine to dinitrogen gas by hydrazine dehydrogenase (HDH). This reaction releases four electrons that are transferred sequentially to: soluble cytochrome c electron carriers (cyt), ubiquinone (Q), the cytochrome bc1 complex (bc1), soluble cytochrome c electron carriers, and, finally to nitrite reductase and hydrazine synthase. This electron transport leads to the translocation of protons from the riboplasm (r, negatively charged) to the anammoxosome (a), resulting in a proton motive force, which is then used to produce ATP by a membrane-bound ATPase [65, 66].

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## Aerobic granules

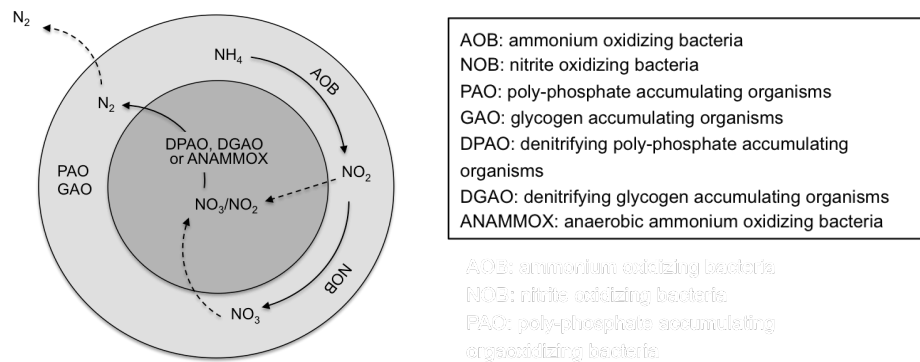
### 1.1.8 Microbial composition

Aerobic granule biological composition and microbial species distribution have been studied by molecular techniques [67]. Bacterial composition, species abundance and distribution could widely vary according to several operational conditions, which could affect the interactions between different bacterial taxa and the ecological niches they occupy. Usually microbial diversity within aerobic granular sludge is strongly related to the composition of feeding medium, seed sludge, operating conditions and size of the granule [14, 68].

Granules are characterized by a concentric multi-layer structure containing channels and pores for the transportation of oxygen and substrates. Aerobic, anoxic and anaerobic zones can be detected along the direction of mass transfer within the granule. This distinctive conformation can provide appropriate growing environments for aerobic, facultative and obligate anaerobic bacteria. Therefore, simultaneous COD, nitrogen and phosphorus removal can be achieved through different redox potentials in the multiple layers of aerobic granules. To date, five different operational microbial taxa have been found in granules sampled at different stages of development [69, 70]:

- Organic compounds oxidizers (heterotrophs);
- Nitrifying (AOB and NOB);
- Denitrifying (e.g., denitrifying glycogen accumulating organisms (DGAO), denitrifying polyphosphate accumulating organisms (DPAO) or ordinary facultative heterotrophs;
- Phosphorus accumulating bacteria (PAO);
- Glycogen accumulating bacteria (GAO).

The granule structure is mainly divided in two parts: an outer aerobic zone, where autotrophic and some of the heterotrophic bacteria (e.g., PAO) are present, and a inner anoxic/anaerobic zone, where denitrifying bacteria (DPAO, DGAO), dead cells and ionic precipitates prevail in the absence of oxygen (Fig. 1.5) [71-73]. Nitrification and aerobic phosphorus uptake occur within the aerobic layer, while denitrification and anoxic phosphorus release can be achieved simultaneously during the aerobic phase in the anoxic zone of the granule by DPAO. The distribution of microbial communities within aerobic granules could result from a difference in growth rate due to competition for space and substrates, and oxygen diffusion limitation across the depth of the granule.



**Figure 1-5. Aerobic granule structure and possible microbial distribution with simultaneous COD, nitrogen, and phosphorus removal**

The presence of nitrifiers has been detected in the aerobic layer at a depth of 70–100  $\mu\text{m}$  from the surface of the granule, while heterotrophs (e.g., PAO and denitrifiers) can exist in both the outer aerobic layer and the centre [74]. Lemaire et al. [74] also stated that in granules larger than 500  $\mu\text{m}$  in diameter, PAO dominate in the outer layers of the granule (200  $\mu\text{m}$ ), while GAO exist in the central layer. Ivanov et al. [75] found a layer of obligate anaerobic bacteria at a depth of 850-1000  $\mu\text{m}$  while dead microbial cells were found at depths greater than 1000  $\mu\text{m}$ .

## Granules cultivation

### 1.1.9 Anammox cultivation and enrichment

As an innovative and more sustainable biological nitrogen removal alternative to traditional nitrification-denitrification technology, the anammox process has the advantages of a higher nitrogen removal rate, lower operational costs, and smaller space requirements. Furthermore, the application of the Anammox process for the treatment of wastewaters characterized by a high content of ammonia and low carbon to nitrogen (C/N) ratio is considered a very attractive alternative to conventional nitrogen removal process. Since its first discovery, anammox-based wastewater treatment processes have been promoted and different reactor types have been developed for this purpose, both at laboratory and pilot-scale. As anammox bacteria grow very slowly, the bioreactor should retain high biomass concentration. For this aim, different bioreactors have been tested for the enrichment of an Anammox culture, including gas-lift reactor, sequencing batch reactor (SBR), fluidized bed reactor and other types. Among these, SBRs are usually recommended [64] because of their efficiency in biomass retention, homogeneity of substrates in the reactor, stability and reliability over a long operational period.

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Various studies showed that several types of sludge, such as activated sludge and sludge taken from UASB and anaerobic digesters could be used as seeding sludge for anammox enrichment, using synthetic wastewater [76]. Finally, in 2002 the first full-scale anammox reactor was run at the sludge treatment plant Sluisjesdijk, Rotterdam, The Netherlands. The reactor, which is fully operating, is fed with partially nitritated sludge liquor from an adjusted nitrification process [77].

It must however pointed out that the start-up of a full-scale anammox reactor could require months or years, and so the availability of a suitable biomass inoculum, and the choice of the anammox bioreactor type are very important to shorten the start-up process. For this reason, usually, the start-up of a new installation is carried out using an inoculum taken from the few existing plants, which apply patented processes such as SHARON-ANAMMOX (SHARON: Single reactor system High Ammonium Removal Over Nitrite, ANAMMOX: ANaerobic AMMonium OXidation), CANON (Completely Autotrophic Nitrogen removal Over Nitrite) and OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) [78].

To date, more than 100 full-scale Anammox references were started up; thus highlighting the deep knowledge regarding the cultivation of this special type of anaerobic granules. Moreover, previous studies regarding anammox cultivation in a laboratory scale Gas-Lift Reactor, have also been successfully conducted by the Department of Civil and Environmental Engineering of the University of Florence. For this reason, this research will focus only on the cultivation of aerobic granules, which represents a newly discovered technology, which still presents few bottlenecks and instabilities.

#### **1.1.10 Aerobic granules cultivation**

Aerobic granular sludge is considered to be one of the most innovative technologies for biological wastewater treatment and since is considered to be a special case of biofilm, one of the main challenges associated with this technology is enhancing the initial cell-to-cell adhesion in activated sludge communities, consequently improving both stability and efficiency of granules.

Aerobic granulation is promoted by different selective pressures applied through operational conditions and is actually affected by several parameters. Therefore, there is a relatively small operational window for a successful cultivation of aerobic granules and to date, one of the most critical parameter remains the poor long-term stability. The stability of aerobic granules means having neither variety in the activity and size distribution of granules, nor granules break up and washout from reactor for a long time. Although aerobic granulation has been

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recently investigated for its advantages over conventional activated sludge systems, further research regarding granules cultivation is still required in order to overcome actual limitations and to develop an efficient and cost-effective technology.

#### **1.1.10.1 Main factors affecting aerobic granulation**

##### *Settling time*

Short settling time is considered one of the most important selective pressures for granulation, especially at early stages. A reduction in the settling time leads to a washout of slowly settling biomass and facilitates the retention of aggregates with higher settling velocities, denser composition and more suitable properties for granulation [79]. Moreover, the washout of suspended bacteria decreases the number of competing microorganisms and promotes biogranulation. Short settling times can also induce EPS production as a microbial response to stressful conditions [80].

##### *Aeration*

Aeration is the main shear stress provider in aerobic granule formation and plays a central role in determining the characteristics of granules. Shear force, in fact, is a fundamental selective pressure for biogranulation influencing the hydrodynamic liquid flow pattern, which can be correlated with microbial aggregation. Actually, aeration, which is usually provided from the bottom of the reactor through an up-flow delivery system, leads to circular flow pattern of the liquid, which will turn aggregations to round and regular shaped granules. [20]. High aeration rates lead to the formation of more compact and denser aggregations characterized by: (1) higher settling velocities; (2) higher biomass concentrations, and (3) an increased protein to polysaccharide (PN/PS) ratio, which is favourable for adhesion of cells and formation of granules.

Low air velocities (i.e.,  $\leq 0.008$  m/s) cannot form granules while medium velocities form large, filament-dominated granules that lead to a failure of the system. Only high air velocities (i.e.,  $> 0.025$  m/s) favour the formation of round and dense granules characterized by higher settling velocities and higher biomass concentrations [81]. High shear forces also increase EPS production and cell hydrophobicity, two important factors affecting granulation [20].

##### *Feast/famine periods*

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Alternating feast and famine periods, characterized by high and low substrate concentrations, significantly influences biogranulation by selecting for slow-growing storing bacteria (e.g., PAO and GAO). These bacteria, in fact, decrease the overall growth rate of the microbial population, which is fundamental to enhance granule long-term stability, and also decrease the cellular hydrophobicity by secreting EPS and therefore promote biogranulation [82]. SBRs are typically the most used type of reactor for laboratory-scale cultivation of aerobic granules, thanks to their ability to alternate feast/famine periods [83]. This feeding strategy promotes the selection for slow-growing bacteria capable to convert the external readily biodegradable carbon source to storage polymers. In fact, during the anaerobic feast phase, accumulating bacteria convert readily biodegradable substrates to slowly biodegradable storage polymers as polyhydroxybutyrate (PHB), while during the aerobic famine period, when no external carbon source is available for conventional heterotrophs, PHB can be used for cell growth and biological phosphorus removal process. The enrichment in PAO or GAO increases EPS production and induces the formation of compact granules [84].

#### *Reactor configuration*

Reactor configuration, especially the height to diameter ratio (H/D), can affect granulation by imposing a selective pressure. A reactor with high H/D ratio (i.e., > 5) would work as a selective device, by increasing the distance the biomass has to travel to be kept inside the reactor during the settling time. Thus, only aggregates with higher settling velocities are retained. Moreover, higher H/D ratios provide longer circular flow patterns during aeration that are necessary for the formation of compact and dense granules. [85] On the other hand, Kong et al. [86] stated that this parameter is not by itself a decisive element for granulation, which, in fact, could vary in full-scale applications. In their study, four reactors with H/D ratios from 4 to 24 with a volume exchange ratio of 50% were run to study the impact of this parameter. An equal percentage of granules with the same microbial structure and size were achieved simultaneously in all four reactors. The results indicate that as long as other selective pressures such as the reduced settling time and high shear stress are provided the configuration of the reactor does not have significant impacts on biogranulation.

#### *Cycle parameters*

In SBRs, cycle time is the sum of the time allocated to each phase occurring in one cycle and defines the HRT of the system as well. Shorter cycle times are thought to be more advantageous for biogranulation. Long cycle times can lead to an increase in the starvation



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phase and cause granular disintegration due to the consumption of EPS [82]. Although cycle time is not considered as a determining factor in granule formation, a reasonable cycle time between 3 and 6 hours is required to allow biomass to grow and accumulate without disintegration or biomass washout [87, 88]. Short cycle times can also negatively affect the granulation process due to:

- The loss of starvation phases required for long-term stability by selecting accumulating bacteria [84];
- Excessive bacterial washout and a decrease in SRT due to solids discharge through effluent withdrawal [89];
- An increase in the organic loading rate by shortening the cycle time could encourage the overgrowth of heterotrophic bacteria retained in the reactor and result in cultivating large and unstable granules [90].

On the other hand, a very long cycle time leads to an increase in the starvation phase, which may cause disaggregation due to the consumption of the EPS by the producers or other microorganisms [82].

#### *Solids retention time*

During the start-up period of aerobic granular sludge processes a variable SRT is present due to high biomass washout from decreased settling times [22, 87]. The SRT of the system can be fixed after full biogranulation is achieved and effluent biomass concentrations are low. SRT can affect the activity of autotrophic and heterotrophic bacteria and consequently the selection of microorganisms within the granules and their ability for nutrient removal and long-term stability. SRT also affects the overgrowth of filamentous bacteria, directly influencing the stability of formed granules [91].

#### *Volume exchange ratio*

The volume exchange ratio can be defined as the ratio of the withdrawn liquid after the settling time to the total working volume of the reactor. High VER (i.e., >60%) imposes a selective pressure by selecting bioparticles with higher settling velocities. Both an increased VER and decreased settling time result in biomass with higher settling velocities and tendencies for EPS production [92, 93]. In systems with identical reactor configuration and operation, higher VERs can impose selective pressure on the system for biogranulation. Most

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aerobic granular sludge processes use VERs of 50% [94]. Wang et al. [92] studied the impact of VER on aerobic granulation in four identical SBRs with the same operating conditions (e.g., settling time, organic loading rate, and aeration intensity) and varying VERs from 20-80%. They observed that under higher VERs (i.e., 80 and 60%) granulation occurred faster and the granules formed were bigger and rounder with lower SVI. Under lower VERs (i.e., 20 and 40%), complete granulation did not occur and a mixture of granules and suspended particles developed in the reactors. Liu et al. [95] introduced the unified selection pressure theory for optimizing the formation and specific characteristics of aerobic granular sludge. According to this theory, the three main selective pressures of settling time, VER, and discharge time can be unified into one parameter called the minimum settling velocity of bioparticles. The calculation for minimum settling velocity considers: (1) the designed settling time; and (2) the settling time that is provided for the biomass during the discharge time.

### *Temperature*

Most aerobic laboratory-scale granulation studies were conducted at room temperature (i.e., 20-25°C). de Kreuk et al. [96] studied the impact of low temperature on aerobic granulation and reported that granules formed slower at low temperatures (i.e., 8°C) compared to higher temperatures (25°C). According to results achieved from the aforementioned study, the bacterial species present in the granule and their growth rate affect the morphology of aerobic granular biomass. Therefore, a variation of the temperature (i.e., seasonal fluctuations for not controlled systems) could affect the bioactivity of the microorganisms and consequently the stability of the granules. Lochmatter and Holliger [94] observed that at temperatures of 20°C the start-up period is shorter, as well as phosphorus removal efficiency is better maintained, than at temperature of 15°C. Song et al. [97] found that granules formed at 30°C are characterized by higher compactness, settleability, and bioactivity compared to the ones cultivated at 25°C and 35°C. Up to now aerobic granulation at low temperature has not been successfully achieved [98]. Granules obtained at low temperature are unstable and readily change to flocculated sludge during settling periods. Flocculation leads to high biomass washout during effluent discharge. de Kreuk et al. [96] reported that granules working at low temperatures could be stable by starting up the reactor at high temperature and then decreasing it after biogranulation has been accomplished.

### *Dissolved oxygen*

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Aeration affects the stability and structure of granular sludge in two ways: inducing the shear force, which is necessary for a successful granule formation; and providing the required DO gradient inside the granule. Granules cultivated under low DO conditions (i.e., saturation under 40%), are usually oversized granules characterized by low density. These properties lead to deterioration and failure of the system. However, de Kreuk et al. [72] found that controlling the DO concentration at very low oxygen saturation levels (i.e., 20%) after the start-up period did not affect the properties of the granules and allowed the highest COD, nitrogen, and phosphate removal efficiencies. Granules cultivated at higher DO concentration (i.e., 2-5 mg L<sup>-1</sup>) are mostly compact and dense, with high settling velocities [99, 100]. According to the literature, DO values at least higher than 2 mg L<sup>-1</sup> during the aerobic phase were favourable for granule formation both in laboratory- and pilot-scale studies [101].

### *Seed sludge*

In most granulation studies the systems are inoculated with conventional activated sludge. Some properties of the seed sludge, such as settleability, surface charge, and hydrophobicity can affect biogranulation. Hydrophobic bacteria are mostly responsible for aerobic sludge granulation, as they are more likely to aggregate together than hydrophilic ones. Therefore, the higher the percentage of the hydrophobic bacteria in the activated sludge, the more favourable the situation for granulation [102]. Therefore, seed sludge originating from biological phosphorus removal systems or from systems with accumulating bacteria would promote the start-up of biogranulation. A recent study by Verawaty et al. [103] showed that a mixture of flocculent sludge and crushed granules enhanced biogranulation by reducing the start-up time and maintaining nutrient removal during the first phases of granulation. In this study, an excessive biomass washout during the first steps of biogranulation was prevented by attachment of flocculent sludge to the crushed granules' surface. This strategy provides an opportunity for the slower growing bacteria responsible for nutrient removal (e.g., PAO and nitrifiers) to be retained in the system.

### *Organic loading rates and carbon sources*

Aerobic granules have been cultivated under OLRs ranging from 0.547 to 13.0 kg Kg COD m<sup>-3</sup> d<sup>-1</sup> [19]. COD removal efficiencies of 70 to 90% indicate how granular systems are capable of withstanding concentrated organic loading rates between 9 and 13 Kg COD m<sup>-3</sup> d<sup>-1</sup>. In most studies high organic strength wastewater has been used, however the use of municipal and other low strength wastewaters have been reported [19, 104, 105]. High organic loading rates

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could increase the diameter of granules by encouraging the growth of heterotrophic bacteria. According to Kim et al. [106] the optimum OLR for aerobic granulation in SBRs is about 2.5 Kg COD m<sup>-3</sup> d<sup>-1</sup>. Other studies have demonstrated that various OLRs can be used to cultivate aerobic granules [19]. Imposing high organic loading rates (i.e., above 6 Kg COD m<sup>-3</sup> d<sup>-1</sup>) under an aerobic feeding mechanism may select for filamentous bacteria and consequently reduce long-term stability [90].

Different type substrates and carbon sources such as easily biodegradable (such as acetate and ethanol), toxic wastewaters (such as phenol and pentachlorophenol), municipal and industrial wastewater (such as dairy and pharmaceutical wastewater) have been used to cultivate aerobic granules. Besides organic loading rate, various carbon sources can influence the strength and microbial composition of granules. Various types of carbon sources can affect the bacteria cells growth and their bioactivity. Changes in the bacteria biological processes can have subsequent impacts on EPS production and composition, which are crucial parameters in granule formation and stability. Cerning et al. [107] observed changes in the EPS production yield by changing the carbon source. They also stated that the biopolymer produced with glucose as the carbon source was different from the one produced in a lactose based media. Moreover, some studies showed that granules cultivated using glucose are more resistant under high OLRs than those cultivated using acetate. At low OLRs glucose-fed granules appear to be loose and filamentous dominated while acetate fed granules are more compact and dense, with a smooth outer layer and better settling velocity [81].

## *Ph*

Even if low pH conditions promote faster biogranulation, usually granules achieved are unstable and fungi-dominated [108]. Low pH values have indeed a strong positive impact on fungi growth and can consequently cause a failure of the entire system [42,108]. Yang et al. [108] reported that granules formed at pH 4 are much bigger (i.e., 7 mm) and looser than those grown at pH 8 (i.e., 4.8 mm). Furthermore, Wan et al. [109] presented pH as the critical parameter for the control of filamentous growth rather than the carbon source. They observed that glucose-fed granules obtained at neutral pH were filamentous-dominated, while acetate-fed ones were mostly compact. By decreasing the pH to 4.5 the acetate-fed granules also showed filamentous overgrowth, whereas by increasing the pH to 8 glucose-fed granules became floc-forming bacteria dominated. Lochmatter and Holliger [94] stated that the optimum pH for reducing the start-up period while maintaining nutrient removal capability is neutral pH. Moreover, it should be taken into account that the proportion of the total non-

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ionised ammonia (free ammonia) is a function of the pH and temperature. In this regard, Yang et al. [110] investigated the role of free ammonia in the development of aerobic granules, pointing out how high pH values could inhibit the activity of nitrifying bacteria due to the pH-enhanced production of free ammonia. The authors concluded that as the free ammonia concentration increased, a significant decrease in cell hydrophobicity and EPS production occurred, preventing the development of aerobic granules.

#### *Filamentous growth*

Filamentous bacteria presence has been commonly detected during aerobic granules cultivation. The definition “filamentous microorganisms” refers typically to heterotrophic and strictly aerobic bacteria but can also include some fungal species, characterized by elongated cells, which appear similar to filaments. When filamentous bacteria concentration is low they do not cause operational problems and may even stabilize the granule structure acting as a rigid backbone [91]. However, once filamentous become predominant in the reactor they can extend from the granule and bind different particles together, increasing the surface area without a corresponding increase in mass. This leads to a decrease in the settleability of the sludge and consequently to a biomass washout from the reactor. This phenomenon is commonly referred as filamentous bulking in aerobic granular sludge. Many factors such as feed composition, substrate concentration, nutrient and dissolved oxygen deficiency, pH and SRT can trigger filamentous growth. Filamentous bacteria are K- strategists, characterised by a lower half-saturation constant ( $K_s$ ) and lower maximum specific growth rate ( $\mu_{max}$ ) than granule-forming ones (r-strategists) and can easily outcompete those bacteria under substrate and nutrient limiting conditions [111]. A high COD/N ratio or a nitrogen deficiency is considered to be one of the main factors triggering bulking [34]. This assumption is confirmed by the kinetic selection theory for filamentous growth. Also, phosphorus, iron, or trace elements deficiency may cause filamentous overgrowth. Most of the past studies focused on aerobic granulation used synthetic feed substrate characterized by a COD/N/P ratio of 100:5:1. It should be noted that due to the low ammonia diffusion coefficient into aerobic granular sludge, a difference between the COD/N ratio in the bulk liquid and the actual COD/N ratio within the granule could be present [91]. Another important factor affecting filamentous growth is dissolved oxygen, since low DO levels may support the proliferation of certain filamentous bacteria species. A DO concentration lower than  $1.1 \text{ mg L}^{-1}$  could promote filamentous growth [112]; therefore a minimum concentration of  $2 \text{ mg L}^{-1}$  is suggested to control the filamentous growth tendencies [111]. DO penetration into the granule’s core is related both to its

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concentration in the bulk solution and to the oxygen consumption rate inside the granule. The DO concentration gradient within aerobic granule can differ significantly from the one present in the bulk solution [91]. Due to their low specific growth rate, a long SRT is favourable for filamentous growth, although some species are able to grow in a wide range of sludge ages. The optimum SRT suggested by Liu and Liu [91] for controlling filamentous bacteria overgrowth in aerobic granular sludge is 10 days; the authors stated that aerobic granules developed at that SRT showed a stable structure, characterized by a good density, smooth outer layers and a low percentage of filamentous organisms. Tay et al. [16] studied the impact of feed composition on granulation and showed that granules fed with glucose-based substrate contained more filamentous bacteria than those fed with acetate-based substrate. It is in fact generally accepted that carbohydrates like glucose, maltose, and lactose and readily biodegradable organic substrates can promote filamentous bulking.

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# **CHAPTER 2**

## **SUITABILITY OF THE GRANULAR SLUDGE FOR THE TREATMENT OF SWINE WASTEWATERS**

## The case study

Modern livestock production is mainly based on indoor systems instead of pasture-based systems, leading to the development of large indoor animal farms. Land limitation and the presence of intensively-raised livestock cause high concentration of animals in confined feedlots and the production of polluted and nutrient-rich wastewaters, which may cause severe environmental issues (eutrophication phenomena, algal blooms and oxygen depletion) if discharged untreated [1].

Swine wastewater is a mixture of urine, faeces and service water containing high concentrations of nitrogen, phosphorous, and organic matter as well as veterinary antibiotics and other toxic compounds. Therefore, the uncontrolled discharge of these wastes could induce a significant negative effect on both the environment and human health, and the use of physical, chemical or biological processes is needed to avoid contamination of soil and water.

A currently adopted swine wastewater treatment scheme is shown in Figure 2.1.

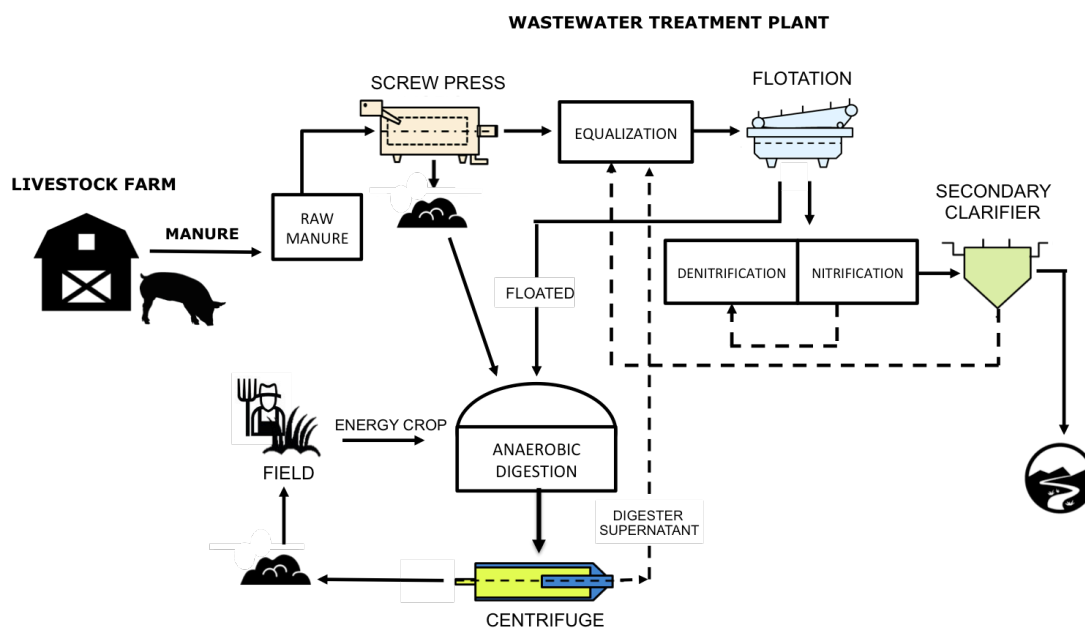


Figure 2-1. Swine wastewater treatment plant scheme.

According to the treatment scheme reported, raw manure coming from the piggery farm is firstly subjected to a preliminary rough solid/liquid separation (screw-press). The originating semi-liquid fraction is then sent to a conventional WWTP, where, after flotation, the clarified water is treated through a conventional activated sludge process. The solid fraction originating from the screw-press step and the floated primary sludge is sent to an anaerobic digester. After the anaerobic digestion process, the digestate is centrifuged: the solid fraction



is used for land spreading (if the application of digested manure is economically suitable and if the required quality management is fulfilled), while the liquid fraction is recycled back to the biological section of the WWTP.

As previously mentioned, despite the common use of activated sludge system, this technology is suffering from some operational dysfunctions and instabilities and the main disadvantages are related to the poor settling property of activated sludge and subsequently to the necessity of a solid-liquid separation after biological treatment, which requires secondary clarifiers with high costs associated to their building and maintenance. Moreover, taking into account the particular matrix of the influent, the liquid fraction of the digested manure, which is recirculated to the biological tank, is very rich in ammoniacal nitrogen but is also characterized by a very low C/N ratio and so requires an external carbon dosage to support the denitrification process. All things considered, the aforementioned treatment process could be implemented by applying two different granular technologies, which could be particularly suitable for both the main- and side-stream manure treatment. Specifically, the poor settling properties of conventional activated sludge could be improved by converting it into a denser and more compact consortium like aerobic granular sludge, while the anammox biomass, which gains their energy for growth from the conversion of ammonium and nitrite into dinitrogen gas, could be used for the treatment of the digester supernatant (Figure 2.2).

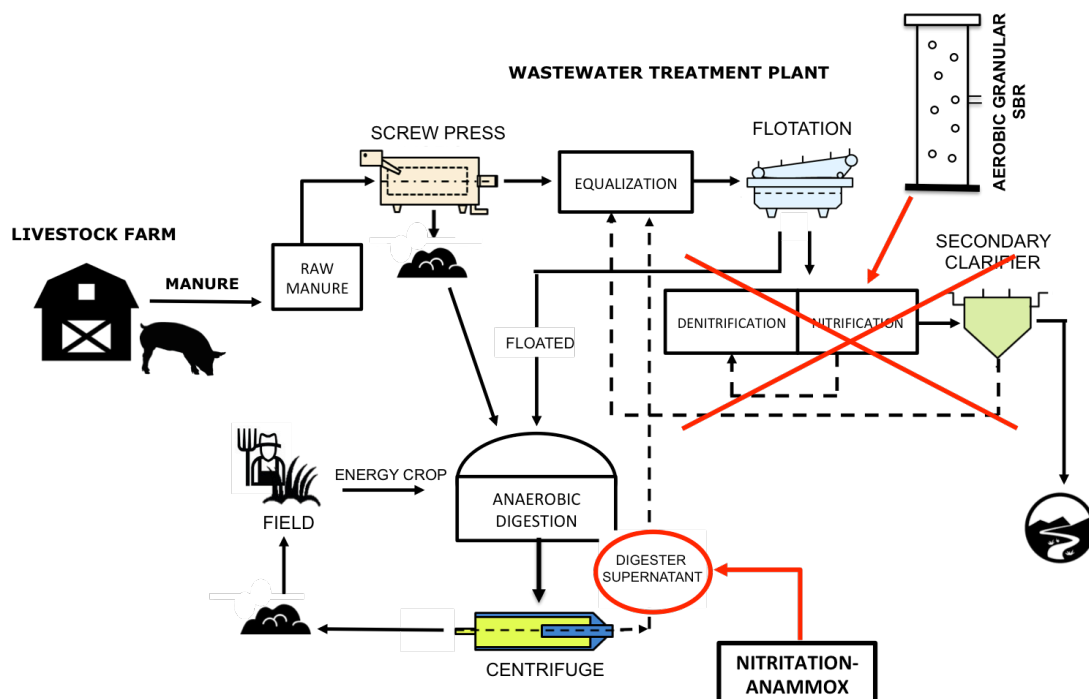


Figure 2-2. Proposal for an alternative manure treatment scheme, which includes anammox process for the treatment of digester liquor and aerobic granules as an alternative to activated sludge.

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The application of aerobic granules for the mainstream treatment can reduce the required space (up to 75%) and capital cost energy (20%) by removing the secondary clarifiers and using SBRs instead of separated bioreactors, according to the capacity of a simultaneous nutrient removal in different zones inside the granules [2]. Moreover, the utilization of the anammox process for the side-stream treatment of digester supernatant instead of conventional nitrification-denitrification process could reduce by over 50% the oxygen demand and by 100% the costs associated to additional external carbon dosage, since no organic carbon is needed. However swine wastewaters may also contain high concentrations of toxic compounds like heavy metals and veterinary antibiotics. The latter are, in fact, prescribed to animals in order to prevent animal infections, cure diseases and as growth promoters [3]. A high percentage of the antibiotics consumed by livestock is excreted unchanged via urine and faeces and is only partially eliminated during the entire wastewater treatment process, so that residual pharmaceutical amounts can explicate their toxic action not only on aquatic and soil organisms, but also on the microbial communities of the plant. Therefore, in order to apply successfully these innovative granular technologies for the treatment of pig slurry, the potential negative effects of the veterinary antibiotics present in these wastewaters on the conversion bioprocesses commonly applied for nutrient removal need to be studied.

## **Occurrence of veterinary antibiotics in the environment**

Pharmaceuticals are a group of contaminants of emerging concern (CEC) widely distributed in the environment, which have remained substantially unidentified until recent advances in low level analytical measurements [4,5]. Pharmaceuticals are classified according to their purpose and antibiotics are defined as naturally occurring, semi-synthetic and synthetic compounds with antimicrobial activity, capable to destroy or inhibit bacterial growth. Antibiotics are used both in human and animal medicine: two-thirds of the European antibiotics consumption is administered for human medicine, while one-third is just related to veterinary application [6], mainly related to poultry and swine livestock therapy [7].

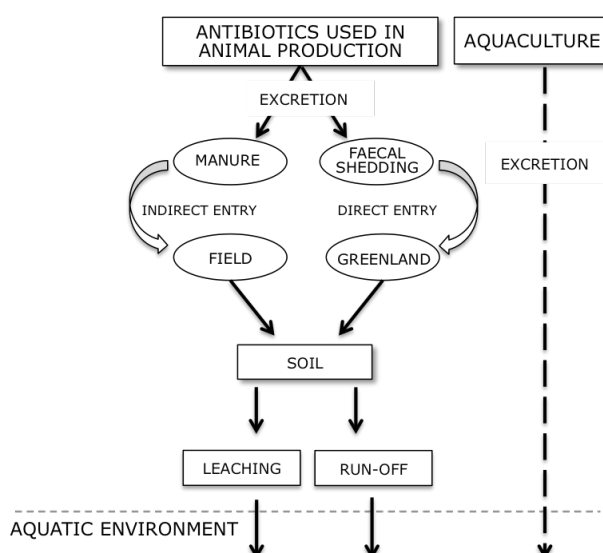
Veterinary antibiotics are mainly prescribed in order to:

- Prevent animal infections;
- Cure animal diseases;
- Enhance growing process.

To date there is no regulation regarding the concentration limits of antibiotics in the

environment, and although their addition to feed as growth promoters in livestock production was banned in Europe in 1998 [8], a massive use of these compounds as drugs is still adopted. Information on their usage in livestock production is also insufficient and this lack of data is alarming because it indicates a veterinary antibiotic administration that is not documented. Besides that, both fate and effects of medical substances in the environment are not clearly identified. The usual pathway for drugs is first to be absorbed by the organism and then be subjected to metabolic reactions. However, many veterinary antibiotics result to be just barely metabolized by the animals, and after conducting an internal curing effect, nearly 30-90% of the parent compound is excreted by urine and faeces as a mixture of metabolites, unchanged substances or conjugated with an inactivating substituent attached to the molecule [9-13]. Conjugates such as acetylated metabolites are inactive and analytically camouflaged, but in manure the acetyl group can be cleaved, releasing the original active ingredient [14]. Antibiotic metabolites can also be bioactive and can be transformed back to the parent compound after excretion [15]. The percentage of excreted antibiotic varies with the antibiotic type, use dosage, specie and age of the animal [16, 17]. Therefore, a significant percentage of the administered antibiotics may enter raw manure in active forms [18, 19].

Three different pathways through which veterinary antibiotics can enter the environment have been fully understood: direct application of antibiotics in aquaculture, direct excretion through urine and faeces of animals kept outdoor and land application of solid or liquid manure as fertilizer (Fig. 2.3) [20].



**Figure 2-3. Veterinary antibiotics exposure pathways in the environment.**

Based upon the Union of Concerned Scientists (UCS) evaluation, specific antibiotics are

administered for different animal species (e.g. cattle, swine and poultry) [21]. Therefore, the occurrence of veterinary antibiotic compounds in the environment is strongly affected by the category of livestock production where the antibiotics come from. Considering either beef, swine and poultry production, the most common classes of antibiotics used and corresponding compounds are listed below [12, 22, 23]:

- Tetracyclines: chlortetracycline, doxycycline, oxytetracycline, tetracycline;
- Macrolides: tylosin;
- Sulfonamides: sulfamethazine;
- Aminoglycosides: lincomycin;
- $\beta$ -lactams: penicillins.

Many investigations have been conducted in order to estimate antibiotics concentration in animal dejecta and manures from large-scale livestock farms [24, 25]. Results showed a significant difference among the sampling areas and the animal species considered and indicated that some antibiotics are present at  $\mu\text{g L}^{-1}$  to  $\text{mg L}^{-1}$ . According to Zhang et al. [26] the residual concentration of selected antibiotics varied in sequence of pig manure > chicken manure > cow manure, and the antibiotics residues were generally higher in animal manures from industrial-scale farms than in those from farmer's households. Typically, antibiotic concentrations in manure are between 1 to 10  $\text{mg L}^{-1}$  but may reach levels up to 200  $\text{mg L}^{-1}$  [12]. The high variability of available data regarding concentration of antibiotics in livestock manure may be due to individual differences in the antibiotics metabolism or to an inadequate extraction and quantification methods used in these studies. Table 2.1 reports the measured concentration of different antibiotics in swine manure.

**Table. 2.1. Concentration of some antibiotics in swine manure.**

Antibiotic	Concentration ( $\text{mg L}^{-1}$ )	Reference
Tetracycline	98	[27]
Oxytetracycline	354	
Chlortetracycline	139	
Doxycycline	37	
Sulfadiazine	7.1	
Tetracycline	30	[28]
Sulphonamides	2	
Tetracycline	5-24	[29]
Chlortetracycline	0.1	
Tetracycline	0.04-0.70	[30]
Sulfamethazine	0.13-0.23	[31]
Sulfathiazole	0.10-0.17	

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Most antibiotics are not biodegradable under aerobic conditions [32-36] and those contained in manure are only partially eliminated in wastewater treatment plants, thus passing unaltered through the system and ending up in the environment, mainly in the water compartments [33]. Biodegradability is very low for the majority of the tested antibiotics, even for some of the  $\beta$ -lactams [37]; only benzyl penicillin (penicillin G) can be completely mineralized [38]. If these substances are partially or negligibly eliminated, they can reach the environment with the potential of adversely affecting aquatic and terrestrial organisms.

Based on previous reports, environmental impacts of residual antibiotics include:

- Induction of bacterial resistance through long-time exposure to antibiotics. A massive antibiotics use can result in selection for resistant bacteria in the gastro-intestinal tract of treated animals, providing a potential reservoir for dissemination of drug resistant bacteria into other animals, humans and the environment. Bacteria have been shown to readily exchange genetic information in nature, mainly through plasmid-mediated process, allowing the transfer of resistance genes from one bacterium to another. This phenomenon can represent a serious threat for public health, as many infections can no longer be treated with the presently known drugs;
- Human health impacts of antibiotic ingestion via animal or plant based food products and via drinking water containing antibiotic residues;
- Toxic effects on aquatic and terrestrial organisms;
- Inhibition of the microbial community involved in wastewater treatment, which may seriously affect organic matter degradation; therefore, effects of antibiotics on these microbial populations are of great interest [33].

Recently, the awareness of all the negative and toxic effects potentially caused by a massive administration of veterinary antibiotics, raised researchers' interest towards a more complex investigation regarding these substances, in order to permit an assessment of the environmental risks they may pose. Within the last decade an increasing number of studies about antibiotic usage, occurrence, fate and effects have been published, but there is still a lack of understanding and knowledge about their pathways in the environment. Further research is then needed to achieve a full understanding of the problem.

## **Application of the anammox process for the side-stream treatment of digested manure**

Anaerobic ammonia oxidation (anammox) is a newly discovered pathway involved in the

microbial nitrogen cycle where ammonia is oxidized to dinitrogen gas, under strictly anoxic conditions, by autotrophic bacteria, consuming nitrite as electron acceptor. As an innovative and more sustainable biological nitrogen removal alternative to traditional nitrification-denitrification technology, the anammox process has the advantages of a higher nitrogen removal rate, lower operational costs, and smaller space requirements. Furthermore, the application of the anammox process for the treatment of digested manure has already been reported [39-41] and also if this treatment scheme has not been applied yet in full scale, it appears suitable for both an environmental and an economic point of view. Otherwise, previous studies demonstrated how several substances, such as substrates (ammonia and nitrite), organic matter (toxic and nontoxic), salts, heavy metals, phosphate and sulphide, can inhibit the anammox biomass, thus reducing its advantages and future application [42]. The exposure to toxic substances can decrease significantly the anammox bacteria activity, thus causing a failure of the system itself since the amount of microorganisms in the reactor will no longer be sufficient to manage the influent nitrogen load, considering the slow growth rate and the low cellular yield.

As previously mentioned, swine wastewater contains high concentrations of veterinary antibiotics, which are not readily biodegradable and remain potentially toxic during the entire wastewater treatment process, partially entering the digestate supernatant. Since antibiotics inhibit microbial activities, they could reduce the anammox bacteria activity and the nitrogen removal rate, negatively affecting the efficiency of digester liquor biological treatment processes.

To date, there are few studies regarding the antibiotic inhibition of anammox bacteria; those that have been published are limited to only four kinds of antibiotics: chloramphenicol,  $\beta$ -lactams, tetracycline and sulphonamides (Table 2.2).

**Table. 2.2. Anammox inhibition due to veterinary antibiotics exposure.**

Antibiotics	Species	Concentration (mg L <sup>-1</sup> )	Activity reduction	Ref.
Chloramphenicol	Chloramphenico l	20	36%	[43]
		200	98% <sup>a</sup>	
		200	68% <sup>b</sup>	
Chloramphenicol	Chloramphenico l	250-1000 <sup>c</sup>	20-80%	[44]
		20 <sup>d</sup>	25%	
Chloramphenicol	Chloramphenico l	320	54%	[45]
Penicillin V	$\beta$ -lactams	100	36%	[43]
Ampicillin	$\beta$ -lactams	400	71%	[43]
		800	94%	
Doxycycline	Tetracyclines	50 <sup>c</sup>	-	[46]

		100	47.6%	
		250	65.1%	
		400	83.2%	
Oxytetracycline	Tetracyclines	25	21%	[47]
		50	52%	
		75	76.5%	
		100	69.5%	
Oxytetracycline	Tetracyclines	92	83%	[45]
Oxytetracycline	Tetracyclines	250 <sup>e</sup>	20%	[3]
		500 <sup>e</sup>	30%	
		1100 <sup>e</sup>	50%	
Oxytetracycline	Tetracyclines	517.5	50%	[48]
Sulfathiazole	Sulphonamide	500	20%	[3]
		650	50%	
		1000	60%	

<sup>a</sup> For the first 3 days of incubation; <sup>b</sup> after the first 3 days of incubation; <sup>c</sup> batch experiment; <sup>d</sup> continuous flow experiment; <sup>e</sup> after 24h of exposure.

In order to apply successfully the anammox process for the treatment of a digester supernatant, the potential negative effect of veterinary antibiotics need to be studied. Therefore, one of the objectives of the present work is to determine the short- and long-term effect on the Anammox process of three veterinary antibiotics (tiamulin, doxycycline and enrofloxacin) commonly administrated to Italian swine livestock, which concentrations and residence times have been previously estimated in the digester liquor of a conventional Italian swine breeding farm [49], so to understand the most suitable conditions to operate this new technology.

## Application of aerobic granules for the main-stream treatment of manure

AGS has been regarded with increasing interest in biological wastewater treatment as it offers solution to the problems common to flocculant biomass; specifically the use of aerobic granules can reduce the required space (75%) and energy (20%) in a full-scale plant by removing the secondary clarifiers and increasing the settling properties of the sludge in a sequencing batch reactor (SBR) configuration instead of separated bioreactors [2]. Moreover, the presence of an extracellular matrix of polymeric substances (EPS) ensures a better resistance to toxic pollutants [50] and makes them particularly eligible for the treatment of antibiotics-containing manure.

Previous studies confirmed that aerobic granular sludge is extremely promising for the treatment of effluents containing toxic compounds (e.g. 2- fluorophenol) [51]; however, little attention was paid to the interaction of antibiotics and granular sludge so far. Since aerobic

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granules represent a novel technology, just few studies focused on evaluating the inhibitory effect of veterinary antibiotics on granules performance and properties. Shi et al. [52] investigated the response of aerobic granules to a long-term exposure to 10 mg L<sup>-1</sup> tetracycline and reported that the presence of the antibiotic led to granules breakup and to an overall deterioration of the reactor nutrient removal performances. Results showed that tetracycline affected both AOB and NOB population, with NOB being the most sensitive. Regarding this results, authors stated that the inhibitory effect of tetracycline might be much higher than the sheltering effect of the granule, concluding that different microbial populations may respond diversely to antibiotics dosage. Amorim et al. [53] investigated the inhibitory effect on the performance and microbial dynamics of an aerobic granular SBR exposed to fluoroquinolones shock loadings. Results obtained revealed that organic removal, as well as nitrifying activity was not affected, although denitrifying and phosphate accumulating organisms' activity were reduced.

Despite few recent studies, there is still a lack of data describing the potential inhibitory effect of these compounds on granular biomass; therefore, in order to achieve an overall perspective regarding the suitability of granular biomasses for the biological treatment of swine wastewaters, a deeper evaluation of the inhibitory effect of veterinary antibiotics on aerobic granular biomass is required.



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# **CHAPTER 3**

## **INHIBITORY EFFECT OF VETERINARY ANTIBIOTICS ON ANAMMOX ACTIVITY**

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## Outline

The suitability of the anammox process for the treatment of swine digester liquor was assessed through the evaluation of the short- and long-term inhibitory effect of three veterinary antibiotics commonly administered to Italian swine livestock. The toxicity of doxycycline, tiamulin and enrofloxacin was evaluated through batch tests designed to estimate the Specific Anammox Activity, expressed as the maximum dinitrogen-gas production over time, related to the biomass concentration in the vials. Moreover, the short-term toxicity of combined concentrations of doxycycline and enrofloxacin was evaluated so to verify whether a synergistic effect could establish. According to the inhibition recorded in presence of the maximum antibiotics concentrations predicted for digester liquor, target compounds do not seem to represent a real hazard for anammox bacteria because at that concentration levels, the activity was just slightly reduced. Moreover, in granular systems inhibition could be easily counterbalanced by increasing the biomass concentration in the reactor, thus assuring the design treatment capacity for antibiotic-rich wastewaters.

## Introduction

Modern livestock production is mainly based on indoor systems clustered in relatively small productive areas. Land limitation and the presence of intensively raised animals in confined feedlots lead to the production of large amounts of polluted and nutrient-rich wastewaters, which may cause negative environmental impacts, if discharged untreated [1]. Among all the manure treatment schemes reported, anaerobic digestion process allows both the reduction of the organic matter fraction and the recovery of a significant amount of energy via biogas production. However, the overall nitrogen content of the produced swine digestate exceeds the amount that can be introduced into the environment, thus requiring an additional treatment. In this scenario, the anammox process, in which ammonium is autotrophically oxidized to dinitrogen gas under anoxic condition, could be applied to treat digestate liquid fraction, that is characterized by high ammonium concentration and low C/N ratio. Anaerobic digestion of swine slurry, followed by a partial nitrification (to oxidize ammonium to nitrite) and the application of the anammox process for the treatment of digester's supernatant has already been investigated [2-4]. Nevertheless, this treatment scheme has not been applied yet in full scale, due to the presence of high concentrations of suspended solids and compounds potentially toxic, such as antibiotics and heavy metals. Moreover, swine wastewater contains high concentrations of veterinary antibiotics administered to animals to prevent infections, cure diseases and promote growth. Veterinary antibiotics represent the most frequent

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pharmaceuticals originating from livestock farming and to date, a massive use of these compounds as drugs is still adopted. Additionally, as many veterinary antibiotics are just barely metabolized by the animals, nearly 30-90% of the parent compound is excreted as a mixture of metabolites, unchanged, or conjugated substances [5, 6]. Consequently, a significant percentage of the administered antibiotics may enter raw manure in active forms, inhibiting the activities of bacteria used for biological treatment. Typically, the most common antibiotics concentrations in manure range from trace levels to 10 mg L<sup>-1</sup>, but occasionally they may also reach concentrations of 200 mg L<sup>-1</sup> [7]. Previous studies demonstrated how several substances, such as substrates (ammonia and nitrite), organic matter (toxic and non-toxic), salts, heavy metals, phosphate and sulphide, can inhibit the anammox biomass, thus reducing its advantages and future application [8]. Specifically, to date, there are few studies regarding the antibiotic inhibition of anammox bacteria; those that have been published are limited to only four kinds of antibiotics: chloramphenicol,  $\beta$ -lactams, tetracycline and sulphonamides [9-15].

Thus, the aim of this work is to determine the short- and long-term effects on the anammox process of three veterinary antibiotics, doxycycline (DOX), tiamulin (TIA) and enrofloxacin (ENR), commonly administered to Italian swine livestock. In fact, the evaluation of the impact of these pharmaceuticals on the anammox activity could facilitate the assessment of the suitability of this innovative process for the treatment of antibiotic-rich digestate liquor.

## **Materials and methods**

### *Inoculum*

As previously described (see Chapter 1, section 1.5.1), anammox cultivation has been extensively studied; moreover, a laboratory scale Gas-Lift Anammox Reactor was successfully started-up by the Department of Civil and Environmental Engineering of the University of Florence. For this reason, this research focuses exclusively on the anammox application, excluding their cultivation.

The granular anammox biomass used for the short- and long-term inhibitory tests (Fig. 3.1) originated from the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam, The Netherlands [16]. The anammox biomass used for the combined short-term tests originated from the full-scale single-stage partial nitrification/anammox (PN/A) WWTP of Olburgen, The Netherlands [17].

The biomass was stored at 4°C, with no substrates and a KNO<sub>3</sub> buffer, so to prevent the production of hydrogen sulphide, and periodically substituted with a new inoculum, in order to avoid microbial decay. The Specific Anammox Activity (SAA), expressed as the maximum dinitrogen-gas production over time, related to the biomass concentration in the vials (gN<sub>2</sub>-N gVSS<sup>-1</sup> d<sup>-1</sup>) of the unexposed biomass was ranging between 0.3 and 0.5 gN<sub>2</sub>-N gVSS<sup>-1</sup> d<sup>-1</sup>. These values are in accordance to those reported in literature [15, 18-22].



**Figure 3-1. Granular anammox biomass**

### Chemicals

The veterinary pharmaceutical products (doxycycline hyclate, tiamulin fumarate and enrofloxacin) were purchased ( $\geq 98\%$  purity) from Sigma-Aldrich (St. Louis, MO, USA). Previous studies estimated the residual concentrations of DOX and TIA in the digester liquor of a conventional Italian swine-breeding farm [23] and assessed the concentration values of ENR present in Italian pigsty sewages [24] (Tab. 3.1). Therefore, the concentration values for each antibiotic were chosen in accordance with these data (Table 3.1).

**Table. 3.1. Percentage of excretion (<sup>a</sup>), estimated concentration in swine wastes (<sup>b</sup>) and tested concentrations (<sup>c</sup>) of the target compounds.**

Antibiotic	% of excretion in active forms <sup>a</sup>	Concentration (mg L <sup>-1</sup> ) <sup>b</sup>	Short-term tests (24-48h) <sup>c</sup>	Long-term tests (9-12d) <sup>c</sup>
Doxycycline	90-95% [19]	12[23] <sup>1</sup>	5-10-50-100 mg L <sup>-1</sup>	50-100 mg L <sup>-1</sup>
Enrofloxacin	90%[20]	0.3-0.5 [24] <sup>2</sup>	25-50-100-200 mg L <sup>-1</sup>	50-100-200 mg L <sup>-1</sup>
Tiamulin	10% [20]	93 [23] <sup>1</sup>	50-100-200-500 mg L <sup>-1</sup>	-

<sup>1</sup> Estimated concentration values in the digester liquor of a conventional Italian swine-breeding farm

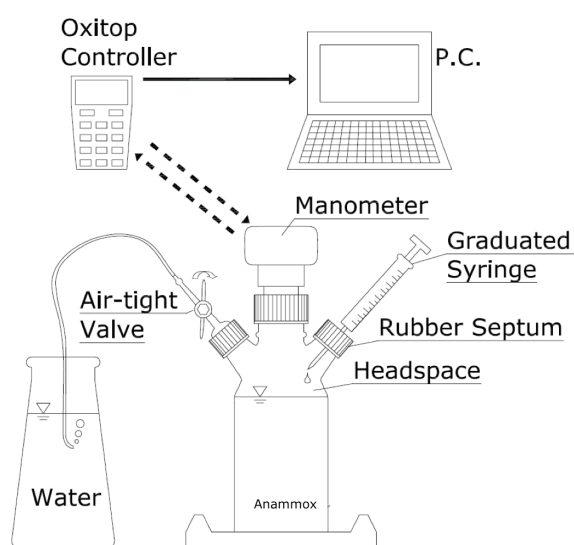
<sup>2</sup> Concentration value in pigsty sewage of an Italian swine-breeding farm.



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### Manometric tests set-up

To determine the short- and long-term effect of veterinary antibiotics on the anammox biomass, batch experiments were performed according to the methodology described by Lotti et al. [25]. Each test consisted of the measurement along the time of the overpressure generated in closed vials, equipped with online manometric sensors (OxiTop Control System; WTW, OxiTop Control AN6, Weilheim, Germany), due to the production of dinitrogen gas, as sketched in Figure 3.2.



**Figure 3-2. Set-up of the manometric equipment for the assessment of the N<sub>2</sub> production rate.**

The manometric devices consisted of 320 mL vials provided with a measuring head with a pressure transducer (sensitivity level 1 hPa) and a data storage system for 360 data points. A detailed description of this procedure can be found in Scaglione et al. [26] and Bettazzi et al. [27]. Manometric determinations of the anammox activity were performed according to the following procedure. The biomass stored at 4°C was firstly washed using dechlorinated tap water in order to remove impurities and cell fragments, and then re-suspended in a medium containing all the microelements needed to avoid nutrient limitation [9]. The pH value of the medium was set to 7.5 with the addition of NaOH or HCl, and 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer to prevent significant pH variations during the test. Each vessel was provided with two lateral holes (Fig. 3.3) sealed with a puncturable rubber septum for both substrate and antibiotics injections and supernatant sampling. The liquid phase (200 mL) and the headspace were flushed with dinitrogen gas through porous stones and needles respectively, in order to guarantee anoxic conditions inside the reactor. The vials were placed in a thermostatic shaker, at 180 rpm and 30°C (Fig. 3.3).

After an initial phase of pressure stabilization (pressure rising as a result of the temperature change), substrates were added by spiked injections through the puncturable septa. The injected *feed solution* contains  $\text{NaNO}_2$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaHCO}_3$  (a concentration of bicarbonate 10 times the stoichiometric need was added to avoid inorganic-carbon limitation during the test) dissolved in high purity water obtained through a milli-Q<sup>TM</sup> system. In the experiments performed the initial ammonium and nitrite concentration was  $50 \text{ mg N L}^{-1}$  ( $100 \text{ mg N L}^{-1}$  as total nitrogen), with nitrite as the limiting substrate.

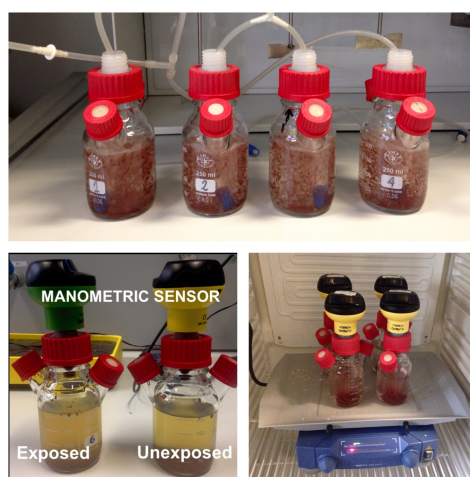


Figure 3-3. Experimental set-up.

After feed injection, the pressure increase due to dinitrogen gas production, and its accumulation in the headspace, was measured and recorded at regular intervals throughout the test. Once the pressure reached a constant value and all nitrite was assumed to be converted (Fig. 3.4), a liquid sample was taken for chemical analysis.

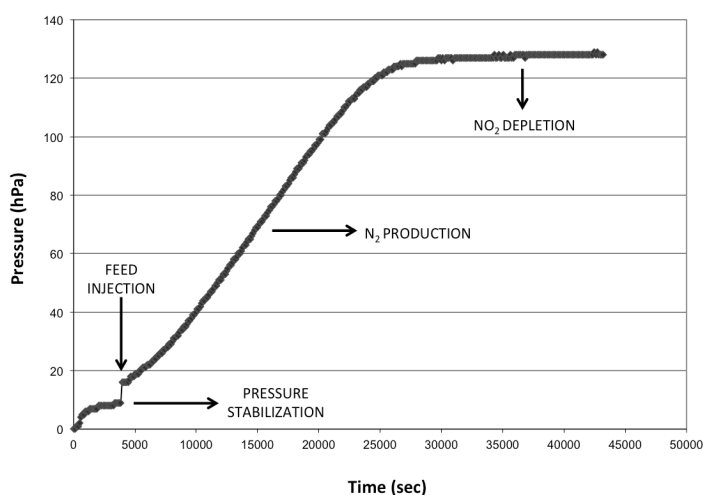


Figure 3-4. Output of a typical manometric tests on anammox biomass.

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This manometric method is based on the principle that the rate of a bioprocess that produces a poorly soluble gaseous component is proportional to the rate of pressure increase. Therefore, dinitrogen gas production can be estimated from the overpressure data during time by applying the ideal gas law:

$$n_{N_2}(t) = \frac{P(t) \cdot V_{hs}}{R \cdot T} \quad [1]$$

Where:

$P(t)$  is the overpressure measured along time by the manometric sensor (KPa);

$V_{hs}$  is the volume of the head space (L);

$R$  is the ideal gas coefficient (L KPa mol<sup>-1</sup> K<sup>-1</sup>);

$T$  is the temperature (K).

For each test, the maximum  $N_2$  production rate  $dN_2/dt$  (mol $N_2$  min<sup>-1</sup>) can be estimated from the maximum slope of the nitrogen production curve.

The maximum Specific Anammox Activity, SAA (g $N_2$ -N gVSS<sup>-1</sup> d<sup>-1</sup>) can also be assessed by referring the nitrogen production rate to the amount of biomass in the bottle:

$$SAA_{max} = \frac{dN_2/dt}{gVSS} \cdot \frac{28gN}{molN_2} \cdot \frac{1440min}{d} \quad [2]$$

This measuring principle was already proven to be applicable and advantageous in the monitoring of anaerobic ammonia oxidation [18, 26, 27] and by comparing the expected stoichiometric  $N_2$  production with that one estimated from pressure data, an average relative error of 4.5±3.3% was assessed [28].

#### *Inhibition tests*

The biomass stored at 4°C was firstly washed with dechlorinated tap water in order to remove impurities and cell fragments, and then the same amount of biomass in terms of wet weight (WW) was distributed in 340 mL bottles filled with 200 mL of a synthetic solutions containing all the microelements needed to avoid nutrient limitation [9] and 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer to prevent pH variations during the

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test. The pH value of the medium was then set to 7.5. The liquid phase and the headspace were flushed with dinitrogen gas, in order to guarantee anoxic conditions inside the bottles. The vials were then placed in a thermostatic shaker at 30°C and 180 rpm. For all the experiments an initial acclimation phase of 6 days was introduced to minimize the stress induced by the transfer from 4°C (temperature of the biomass storage) to 30°C. During this phase, bacteria were fed daily and their activity gradually increased. Once the activity reached a stable value, a pulse of concentrated stock solution of the target antibiotic was added. At the end of the exposure time, substrates ( $\text{NaNO}_2$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaHCO}_3$ ) were added by spiked injection and the pressure increase was recorded at regular intervals throughout the test. Regarding the long-term inhibitory tests, the dilution of the initial antibiotic concentration due to repeated injections of *feed* solution (1 mL a day) as well as the bacterial growth due to substrate consumption, were quantified. For the longest experiment, 6% dilution and a 5% increase in VSS concentration due to biomass growth was calculated, respectively, thus excluding an underestimation of the inhibitory effect of the target compounds. The initial ammonium and nitrite concentration was 50 mg N L<sup>-1</sup> each (100 mg N L<sup>-1</sup> as total nitrogen), with nitrite as the limiting substrate; as the pressure reached a constant value and all nitrite was assumed to be converted, a liquid sample was taken for chemical analysis. The percentage of activity maintained by anammox bacteria after the exposure to different antibiotics concentrations was calculated with respect to the average activity of the unexposed biomass (control).

### *Analytical methods*

Ammonium, nitrite and nitrate were determined spectrophotometrically using commercial test kits according to the protocol of the manufacturer (brand: Dr.Lange test kits, Hach-Lange GmbH, Düsseldorf, DE; kits LCK 303 for ammonium, LCK 341 for nitrite and LCK 339 for nitrate) and determined on a designated spectrophotometer (Dr. Lange 3600). All liquid samples were filtered at 0.45 µm before analysis. The concentrations of solids as Total Suspended Solids (TSS), and the fraction corresponding to the biomass as Volatile Suspended Solids (VSS), were determined according to the Standard Methods [29].

## **Results and discussion**

### *Short-term effect of doxycycline, tiamulin and enrofloxacin*

The short-term effect of DOX, TIA and ENR on the anammox activity has been evaluated

through batch tests and the concentrations tested are reported in Table 3-1. Results reported, comprising those achieved from specific short-term tests and those obtained from the initial part of the long-term tests, are shown in Figure 3.5, 3.6 and 3.7 respectively.

Figure 3.5 shows the percentage of the average activity left of the biomass exposed to different DOX concentrations compared to controls (black dotted line). Each data point represents the average of two replicates. The activity of the exposed biomass was always compared to that of the unexposed biomass measured the same day, so to prevent an activity decrease due to other variables than the antibiotic exposure.

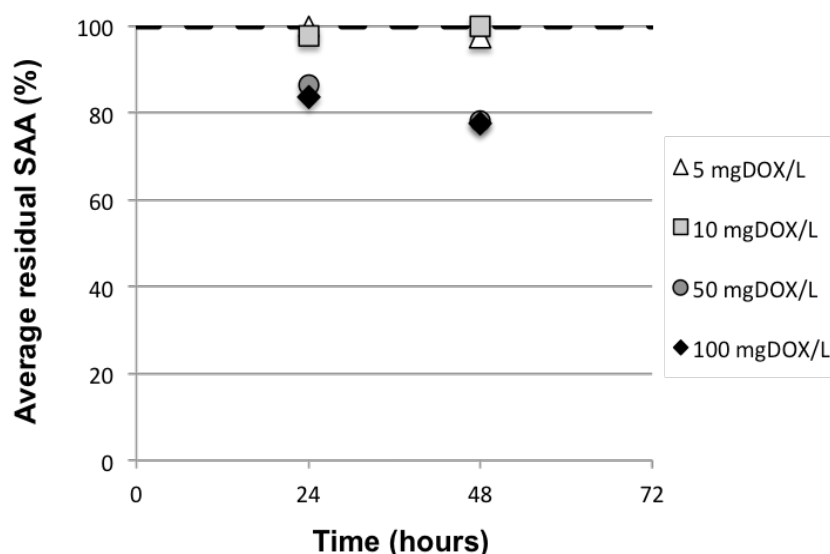


Figure 3-5. Average SAA left evaluated after 24 and 48h exposure to DOX concentrations of 5, 10, 50 and 100 mg L<sup>-1</sup>. 100% represents the activity of anammox-unexposed culture.

Despite the fact that at 5 and 10 mg L<sup>-1</sup> DOX no inhibition is detected after 48 hours, the effect observed in presence of 50 mg L<sup>-1</sup> and 100 mg L<sup>-1</sup> DOX is nearly similar, with an initial 14-17% inhibition after 24 hours, followed by a further activity reduction of 22% after 48 hours. Although the short-term inhibitory effect of 50 and 100 mg L<sup>-1</sup> DOX is similar, it increases with longer exposure time. Figure 3.6 exhibits the percentage of the average activity left of the biomass exposed to TIA compared to controls (black dotted line).

From data acquired TIA activity increases with higher concentrations and longer exposure time. A negligible loss in activity (5%) is calculated at concentrations of 50 mg L<sup>-1</sup> TIA after 24 and 48 hours, whereas higher concentrations lead to further decreasing activities: after 48 hours exposure to 100 and 200 mg L<sup>-1</sup> TIA, the anammox activity decreases by 18 and 21% respectively. A significant 64% decrease of activity occurs after 48 hours exposure to 500 mg L<sup>-1</sup>.

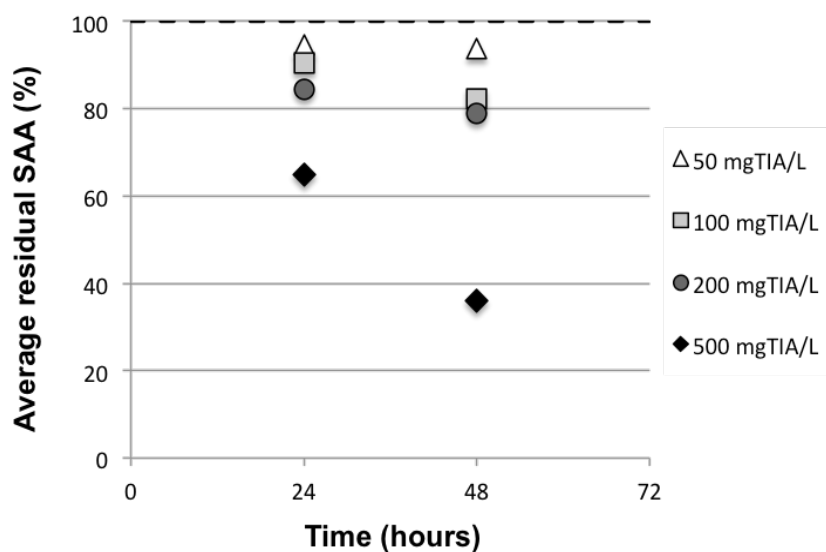


Figure 3-6. Average SAA left evaluated after 24 and 48h exposure to TIA concentrations of 50, 100, 200 and 500 mg L<sup>-1</sup>. 100% represents the activity of anammox-unexposed culture.

Figure 3.7 reports the percentage of the average activity left of the biomass exposed to ENR compared to controls (black dotted line). The short-term inhibitory effect of ENR increases with higher concentrations, but remains approximately stable with the increase of exposure time from 24 to 48 hours.

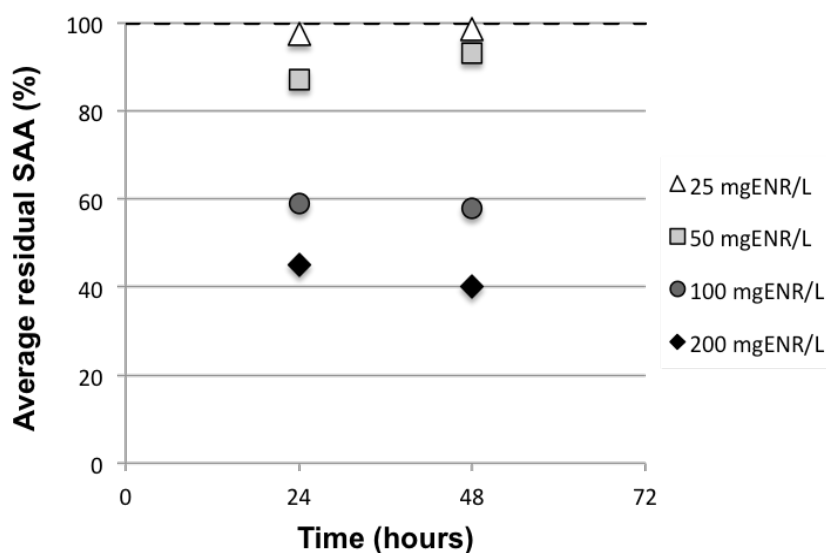


Figure 3-7. Average SAA left evaluated after 24 and 48h exposure to ENR concentrations of 25, 50, 100 and 200 mg L<sup>-1</sup>. 100% represents the activity of anammox-unexposed culture.

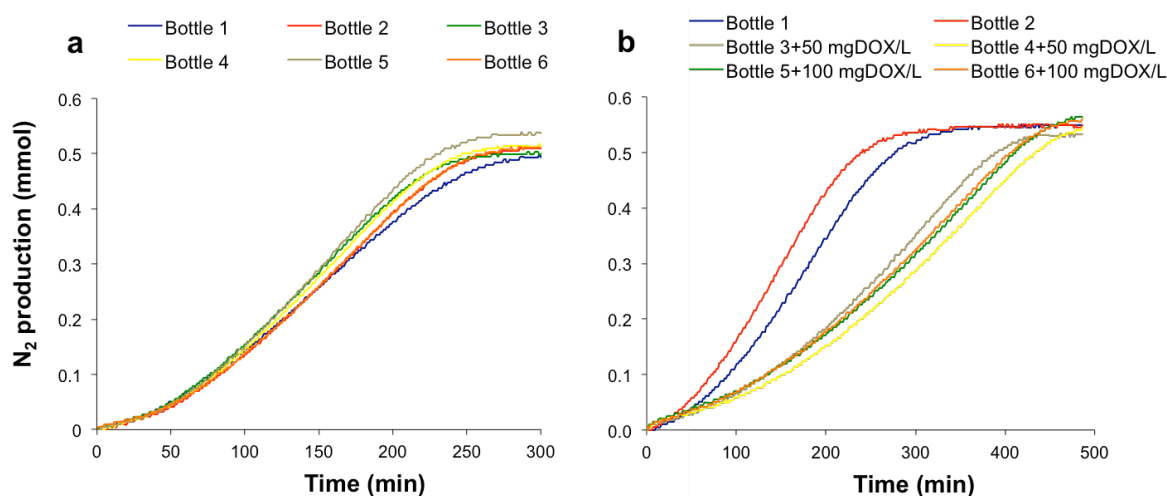
At concentrations of 50 mg L<sup>-1</sup> ENR, 10% inhibition occurs after 24-hour and this value is similar to that obtained for DOX. On the contrary, DOX inhibition increases with longer exposure time, while the inhibition caused by the exposure to ENR is halved after 48 hours. Considering

higher concentrations, the activity is increasingly reduced by 42% and 60% after 48 hours exposition to 100 and 200 mg L<sup>-1</sup> ENR respectively. From the comparison between the inhibition recorded in presence of the lowest concentration tested for each antibiotic (50 mg L<sup>-1</sup>), the short-term inhibitory effect of DOX resulted to be stronger than that due to ENR and TIA and varied in sequence of DOX > ENR > TIA. The sensitivity of anammox bacteria towards DOX was also highlighted by Alvarino et al. [12]. In the referred study the inhibitory effect of two veterinary pharmaceuticals (acetaminophen and DOX) was evaluated for different types of biomass involved in the nitrogen removal processes via nitrite, with anammox bacteria being significantly inhibited at high DOX concentrations. Noophan et al. [13] studied the short-term inhibitory effect of oxytetracycline, confirming a partial reduction of the anammox activity at concentrations of 23-100 mg L<sup>-1</sup> of the target compound.

#### *Long-term effect of doxycycline and enrofloxacin*

Based on the results achieved from the short-term inhibitory tests, the effect of prolonged exposition was evaluated for those antibiotics that exerted the higher short-term inhibition on anammox activity: DOX and ENR. The long-term exposure effect was evaluated by repeated batch assays conducted for 12 (DOX) and 9 (ENR) days at regular intervals of 24-hour. For both the experiments an initial acclimation phase was introduced so to minimize the stress induced by the transfer from 4°C (temperature of the biomass storage) to 30°C.

Dinitrogen gas production rate for each bottle before DOX addition and after 6-day exposure to 50 and 100 mg L<sup>-1</sup> (when the maximum activity reduction was detected) is reported in Figure 3.8.

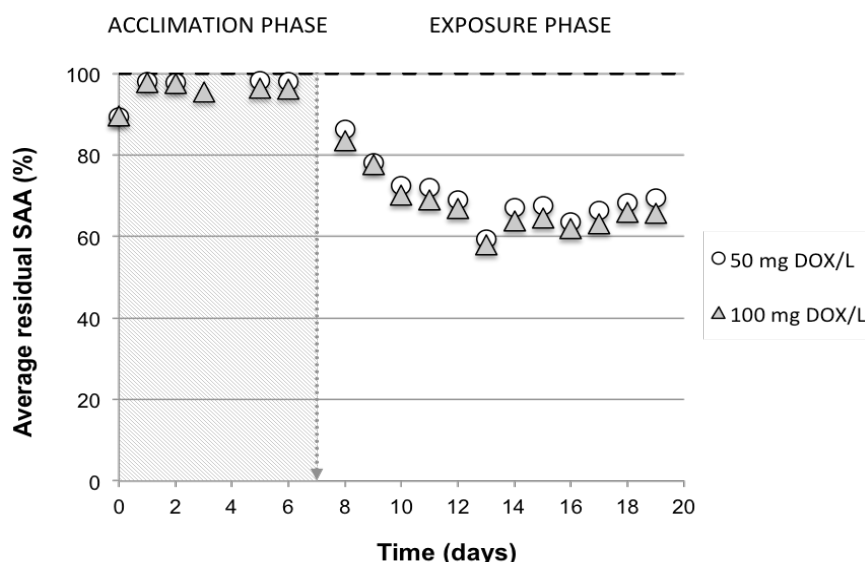


**Figure 3-8.** Dinitrogen gas production rate before and after 6-day exposure to different DOX concentrations.

Figure 3.9 shows the average percentage of residual activity of the biomass exposed to DOX compared to controls (black dotted line) before and after the antibiotic injection.

According to the experimental data obtained, DOX concentrations of 50 and 100 mg L<sup>-1</sup> DOX induced similar inhibitory effects, whose higher values (41% and 42% activity reduction) were registered after 6 days of exposure (Figure 2a). After this activity decrease a partial recovery occurred, leading to inhibition values of 31% and 34% for biomass exposed to 50 and 100 mg L<sup>-1</sup> DOX respectively.

These results are similar to those found by Alvarino et al. [12] for DOX concentrations of 100 mg L<sup>-1</sup>. In the referred study the exposure to 100 mg L<sup>-1</sup> DOX reduced the anammox activity by 47.6% with an estimated IC<sub>50</sub> value for this pharmaceutical of 121 mg L<sup>-1</sup>.



**Figure 3-9. Average SAA left evaluated before and after the exposure to DOX concentrations of 50 and 100 mg L<sup>-1</sup>. 100% represents the activity of anammox unexposed culture.**

A concentration- and exposure time-dependency of tetracyclines action on anammox biomass was as well found by Lotti et al. [15]. However, the authors registered an anammox activity reduction at oxytetracycline concentration of 100 mg L<sup>-1</sup> only after 7 days, thus indicating a delayed inhibitory effect of oxytetracycline compared to that due to DOX.

Dinitrogen gas production rate for each bottle before ENR addition and after 9-day exposure to 50, 100 and 200 mg L<sup>-1</sup> is reported in Figure 3.10.



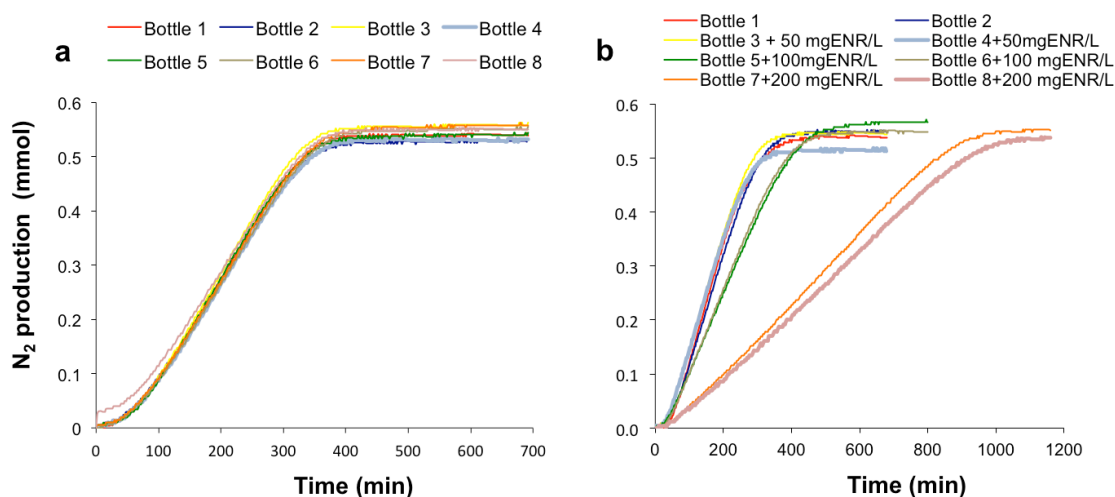


Figure 3-10. Dinitrogen gas production rate before and after 9-days exposure to different ENR concentrations.

Figure 3.11 shows the average percentage of residual activity of the biomass exposed to ENR compared to controls (black dotted line) before and after the antibiotic injection. In presence of  $50 \text{ mg L}^{-1}$  ENR, an initial 13% inhibition occurred after 24 hours, followed by a complete activity recovery after 5 days (Figure 2b). Exposure to higher concentrations caused an increasing reduction of the activity; after 2 days at  $100 \text{ mg L}^{-1}$  ENR the activity is reduced by 42%, followed by a partial recovery, which ends in an activity reduction of 30% after 9 days. Concentrations of  $200 \text{ mg L}^{-1}$  ENR reduced additionally the activity by 80% after 9 days.

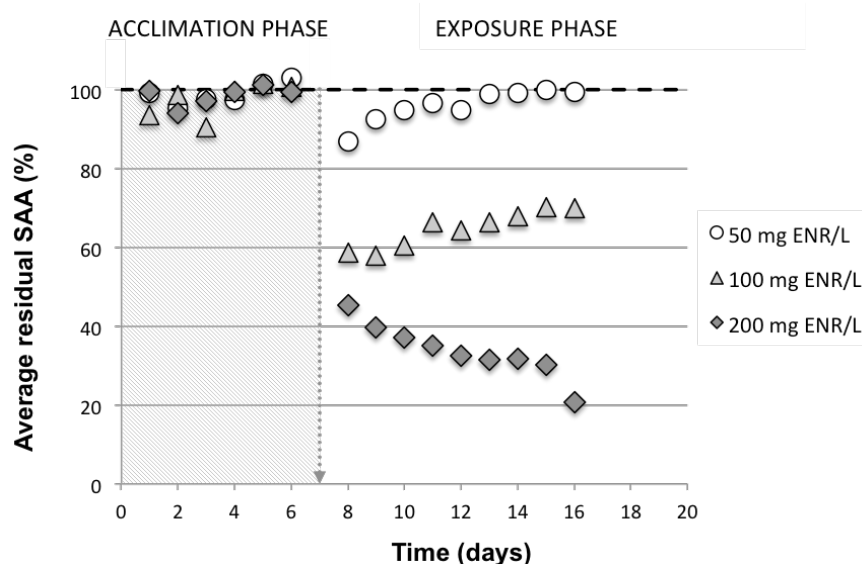


Figure 3-11. Average SAA left evaluated before and after the exposure to ENR concentrations of 50, 100 and  $200 \text{ mg L}^{-1}$ . 100% represents the activity of anammox unexposed culture.

These data confirm that fluoroquinolones exhibit an inhibitory effect strictly dependent on the concentration of the chemical [30, 31] and so toxicity becomes more pronounced as the drug

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concentration increases. To date, ENR action has not been assessed for anammox biomass, but the influence of different concentrations of ciprofloxacin was investigated in four partial-nitrification bench-scale bioreactors [32]. The addition of 100 ng L<sup>-1</sup> of ciprofloxacin induced a temporary 40% reduction of the ammonium oxidizing bacteria (AOB) activity and of AOB concentration in the system, even though the toxic effect completely disappeared within a month, suggesting that AOB can adapt to low ciprofloxacin concentrations. Furthermore, the addition of 350 ng L<sup>-1</sup> decreased significantly the partial-nitrification process rate, resulting in a 60% reduction of the biomass concentration inside the reactor. Moreover, a deep change in the microbial composition was detected, indicating that ciprofloxacin affected drastically the AOB population and selected for microorganisms resistant and/or degraders of this antibiotic. These results correspond to those obtained in the present study for ENR; although concentrations tested were much higher, the same activity recovery for the low concentrations tested was observed, together with an increasing toxicity for high concentrations.

Comparing the long-term effect of DOX and ENR the maximum inhibition occurred in presence of 50 mg L<sup>-1</sup> DOX (41%) is higher than that due to the same ENR concentration (13%), but these inhibition values were registered respectively after 6-day and 24-hour exposure, thus indicating that the inhibitory effect of ENR reaches its maximum shortly after the administration. These results are in accordance with those obtained from the short-term tests in terms of the higher toxicity of DOX compared to ENR. The faster mechanism of action exerted by ENR was also confirmed by the inhibition values detected at concentrations of 100 mg L<sup>-1</sup> of the target compounds. Even though a maximum inhibition of 42% occurred for both antibiotics, these values were registered after 6 days for DOX and after 2 days for ENR.

A possible explanation for the faster mechanism of action exerted by ENR could be ascribed to the molecular basis of tetracyclines and fluoroquinolones transport inside the cell, which may involve channel forming proteins [33], whose genes have been detected also in anammox bacteria [34]. Anyway, taking into account the lack of data regarding the molecular basis of antibiotics transport and the influence of this pharmaceutical on the anammox biomass, further research is needed so to explain in more detail the basis of the differences in the mechanism of action of ENR and DOX. According to the target pharmaceuticals concentrations measured in swine wastewaters the presence of tested antibiotics does not seem to represent an obstacle for the application of the anammox process for the treatment of digester supernatant, since at that concentration levels, just a negligible inhibition of the microbial activity was registered. Moreover, in case of an exceptional antibiotics dosage, the process could also rely on the overcapacity distinctive of biofilms (granular). The overcapacity of

biofilm systems derives from the presence of steep concentration gradients along the biofilm thickness, resulting in large part of the biomass operating under stringent substrate limiting conditions. Furthermore, the good settling properties facilitate biomass retention and thus the maintenance of long SRTs [35,36].

#### *Short-term combined doxycycline and enrofloxacin test*

Since swine wastewaters are a mixture of different veterinary antibiotics, after the evaluation of the short- and long-term effect caused by the single administration of different antibiotics, the effect of combined concentrations of DOX and ENR was evaluated in order to verify if a synergistic effect (effect that is greater than the algebraic sum of individual effects) could establish. The tested concentrations of both antibiotics are shown in Table 3.2; the short-term effect has been assessed for an exposure time of 24, 48 and 72 hours. Inhibition values registered are shown in Table 3.3.

**Table. 3.2. Concentrations of DOX and ENR tested simultaneously.**

		Enrofloxacin mg L <sup>-1</sup>				
		0	25	50	75	100
Doxycycline mg L <sup>-1</sup>	0	-	X	X	X	-
	25	X	X	X	-	-
	50	X	X	X	-	-
	75	X	-	-	X	-
	100	-	-	-	-	X

Results achieved indicate that the inhibitory effect cause by the simultaneous addition of DOX and ENR increases with higher concentration and longer exposure time.

Regarding the percentage of inhibition due to the single administration of the target compounds, during this experiment, DOX exerted a higher inhibition than that registered for the short-term tests. The difference in the effect exerted by DOX in these experiments, may be due to the type of anammox biomass used, which originated from the full-scale CANON-anammox reactor of Olburgen instead of Dokhaven-Sluisjesdijk, as the biomass used for the short-term test. This new biomass was characterized by lower anammox enrichment and by lower specific activity ( $0.348 \pm 0.01$  vs  $0.417 \pm 0.008$ ), due to the large fraction of ammonium

oxidizing biomass; however, also in this case the inhibition registered for the antibiotics concentrations foreseen for digester liquor, would not affect significantly microbial activities.

**Table. 3.3.** Inhibition registered for different combinations of DOX and ENR concentrations after 24 (<sup>a</sup>), 48 (<sup>b</sup>) and 72 hours (<sup>c</sup>). The occurrence of a synergistic effect is disclosed in bold text.

	Enrofloxacin mg L <sup>-1</sup>						
Doxycycline mg L <sup>-1</sup>		0	25	50	75	100	
	0	-	2.5 <sup>a</sup> 1.3 <sup>b</sup> 0.9 <sup>c</sup>	7.0 <sup>a</sup> 6.0 <sup>b</sup> 7.1 <sup>c</sup>	34.7 <sup>a</sup> 28.7 <sup>b</sup> 33.9 <sup>c</sup>	-	
		25	22.2 <sup>a</sup> 26.2 <sup>b</sup> 36.1 <sup>c</sup>	23.7 <sup>a</sup> <b>38.6<sup>b</sup></b> <b>48.1<sup>c</sup></b>	27.1 <sup>a</sup> <b>41.0<sup>b</sup></b> <b>56.0<sup>c</sup></b>	-	-
			50	28.7 <sup>a</sup> 35.8 <sup>b</sup> 48.1 <sup>c</sup>	<b>36.3<sup>a</sup></b> <b>51.2<sup>b</sup></b> <b>66.3<sup>c</sup></b>	<b>36.2<sup>a</sup></b> <b>57.3<sup>b</sup></b> <b>69.5<sup>c</sup></b>	-
	75			38.4 <sup>a</sup> 40.4 <sup>b</sup> 50.6 <sup>c</sup>	-	-	55.5 <sup>a</sup> 65.6 <sup>b</sup> 70.9 <sup>c</sup>
		100					

A synergistic effect can be detected after 48 and 72 hours for the following concentrations (Table 3.3):

25 mg L<sup>-1</sup> DOX + 25 mg L<sup>-1</sup> ENR

25 mg L<sup>-1</sup> DOX + 50 mg L<sup>-1</sup> ENR

and after 24, 48 and 72 hours for the following concentrations:

50 mg L<sup>-1</sup> DOX + 25 mg L<sup>-1</sup> ENR

50 mg L<sup>-1</sup> DOX + 50 mg L<sup>-1</sup> ENR

Actually, a synergistic effect of DOX-ENR has been documented neither for anammox bacteria, nor for other genera involved in nitrogen removal. However, Yang and Jin [37] evaluated the combined effect of oxytetracycline and copper (II) and oxytetracycline and sulfide (S<sup>2-</sup>) on anammox biomass. According to the results obtained, the joint toxicity of oxytetracycline and copper (II) on the anammox mixed culture resulted to be antagonistic, whereas the interaction between oxytetracycline and sulfide was synergistic. Katsou et al. [38] evaluated the inhibitory

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effect caused by the combined use of tetracycline, doxycycline and amoxicillin on AOB and nitrite oxidizing bacteria (NOB) activity. On the one hand, the authors reported that the combined use of 250 mg L<sup>-1</sup> amoxicillin & 250 mg L<sup>-1</sup> tetracycline and 250 mg L<sup>-1</sup> doxycycline & 250 mg L<sup>-1</sup> amoxicillin reduced AOB activity more than the use of 500 mg L<sup>-1</sup> of each substance separately. On the other hand, the combined administration of 250 mg L<sup>-1</sup> tetracycline & 250 mg L<sup>-1</sup> doxycycline caused a smaller inhibition of AOB and NOB activity than the single use of 500 mg L<sup>-1</sup> tetracycline.

Although the inhibition caused by the single use of the maximum DOX and ENR concentrations expected for digester liquor suggests that target compounds do not represent a critical threat for the anammox process, after 72 hours exposure to combined concentrations of 25 mg L<sup>-1</sup> of either antibiotics a 48% inhibition was recorded. Since these concentration values exceed those commonly present in digester supernatants, presumably no significant interference would occur at expected concentrations. Moreover, the activity reduction due to a temporary, massive antibiotics administration could be potentially overcome by the overcapacity characteristic of granular systems.

## Conclusions

The inhibitory effect of three veterinary antibiotics commonly administrated to Italian swine was investigated. Results show that the short-term inhibition due to the exposure to DOX and TIA increases with higher concentrations and exposure time, while the short-term inhibitory effect of ENR increases with higher concentrations, but remains almost stable during time. Taking into account the percentage of inhibition registered in the presence of the lower concentration tested for all the antibiotics, the inhibitory effect of DOX results to be stronger than that due to TIA and ENR, and varies in sequence DOX > ENR > TIA. Long-term DOX toxicity was confirmed to be stronger than ENR, which exhibited concentration-dependent bacterial inhibition and a faster mechanism of action. Finally, a synergistic effect was detected for combined concentrations of DOX and ENR, thus leading to an effect greater than the sum of individual effects and highlighting the importance of this phenomenon.

Although the inhibition values caused by the single administration of the maximum antibiotics concentrations foreseen for digester liquor suggest that the application of PN/A process for the treatment of these effluents seems conceivable, after 72 hours exposure to combined concentrations of 25 mg L<sup>-1</sup> of either antibiotics a 48% inhibition is recorded. Since concentrations tested exceed those commonly present in digester supernatants, presumably no interference would occur at expected concentrations. Moreover, the activity reduction due

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to a temporary, massive antibiotics administration could be potentially overcome by the overcapacity characteristic of granular systems.

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# **CHAPTER 4**

## **AEROBIC GRANULATION IN A SEQUENCING BATCH REACTOR: GRANULES FORMATION AND NUTRIENT REMOVAL CHARACTERISTICS**

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## Outline

Aerobic granular sludge is considered to be one of the most innovative technologies for biological wastewater treatment according to its several advantages over conventional activated sludge systems, which can result in a compact and cost-effective full-scale application for domestic and industrial wastewater treatment. Contrary to anaerobic granules cultivation, the cultivation of aerobic granules still presents some drawbacks, which need to be overcome in order to convert laboratory-scale attempts into an innovative cost effective full-scale process. During the last decades, the characteristics of aerobic granule structure and the factors affecting biogranulation process have been investigated, in order to understand the most advantageous operating conditions and to improve granules formation and long-term stability. Nevertheless, the full-scale application of this technology still presents some drawbacks, mainly related to the poor stability of granules, which makes the reactor performance unreliable. To date, granules break up and washout phenomena have not been fully understood; therefore, a deeper knowledge of the causes interfering with granules stability is fundamental for the development of suitable full-scale aerobic granules-based bioreactor. This chapter comprises a preliminary study, aimed at evaluating factors affecting the bio-granulation process under different conditions, and describes the start-up of an aerobic granular SBR with the intent to achieve the *inoculum* for the subsequent inhibitory tests, aimed at evaluating the toxicity of different toxic compounds on this type of biomass. In order to assess under controlled laboratory conditions the effect of selected pharmaceuticals on unacclimated biomass, domestic sewage was chosen as reactor influent. Moreover, this solution was also adopted in order to compare the susceptibility previously measured for the anaerobic (anammox) granules originated from the municipal full-scale reactor of Dokhaven–Sluisjesdijk with that measured for aerobic granules.

## Introduction

AGS is considered to be a paradigm shift in biological suspended growth wastewater treatment due to: elimination of universal problems of settling as granules are dense and compact and settle rapidly under all conditions; elimination of the need for multi-redox potential zones necessary in conventional biological nutrient removal; high efficiency in conditions of variable strength wastewaters. The granular systems retain high biomass concentrations in the reactor by increasing the substrate conversion capacity, enhance solid-liquid separation due to high settling velocity of the granules, and achieve better effluent quality than conventional activated sludge systems [1]. Besides that, the robust microbial

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structure leads also to the ability to withstand high organic loading rates, while the presence of extracellular polymeric substances (EPS) matrix ensures a better resistance to inhibitory and toxic pollutants [2]. Aerobic granules are characterized by a concentric multi-layered structure containing channels and pores for the transport of oxygen and substrates [3]. Aerobic, anoxic, and anaerobic zones characterized by different redox potentials can be defined along the direction of mass transfer within the granule. The different redox potentials allow for simultaneous COD, nitrogen, and phosphorus removal by encouraging the growth of aerobic, facultative, and obligate anaerobic bacteria [4]. Aerobic granulation is a gradual process initiating from flocculent seed sludge to compact aggregates, then to granular sludge and finally to mature granules, without using any carrier materials [5]. The interaction between bacteria includes repulsive electrostatic force, attractive van de Waals force and repulsive hydration interaction, so for cells to aggregate many conditions have to be satisfied in order to promote microbial adhesion [6]. Therefore, the main challenges associated with this technology are enhancing the initial cell-to-cell adhesion and improving the stability of granules during the practical application of this technology. Granules instability is, in fact, one of the major problem, which leads to granules breakage and to a massive solids discharge in the effluent and to date it has not been fully resolved. Moreover, it can also affect carbon and nutrient removal efficiency, thus causing a violation of the effluent limits and a failure of the system itself.

Aerobic granules are usually cultivated in Sequencing Batch Reactors (SBR), in which granulation is promoted by the application of varying selective pressures. To date the influence of many factors (such as hydrodynamic shear force, settling time, volume exchange ratio, hydraulic retention time, extracellular polymeric substances, dissolved oxygen, organic loading rate and feeding strategy) on this process have been studied, in order to understand the most advantageous operating conditions. The cycle set-up of a SBR consists of a filling, aeration, settling and effluent discharge phase; the settling time and the percentage of working volume discharged at the end of each cycle are considered as crucial parameters for the removal of flocculent biomass from the reactor. Another critical parameter to enhance granule long-term stability is the growth rate of the microorganisms that are part of it: the lower the growth rate, the higher the stability [1]. Heterotrophs growth rate decreases when they grow on slowly biodegradable stored polymers, instead of easily biodegradable carbon sources [7]. Therefore, the overall growth rate of the microbial population can be decreased by selecting for slow growing organisms (such as PAO and GAO), able to convert all easily biodegradable substrates to slowly biodegradable storage polymers (such as PHA and glycogen). The establishment of an anaerobic feeding phase and the application of a feast-

famine regime would promote the conversion of the external readily biodegradable carbon source to storage polymers. Aim of this work is the investigation at laboratory-scale of the aerobic granulation process and the start-up of a Sequencing Batch Reactor with the aim of translating first observations into a stable and economically sustainable full-scale system.

## Preliminary granulation study

### 4.1.1 Materials and methods

#### *Experimental set-up*

Two column-type reactors (R1 and R2) of 60 cm in height, 12 cm in diameter and total working volume of 5.7 l were operating as sequencing batch reactors, with volume exchange ratio of 50%. The reactors were operating sequentially in 4-hour cycle comprising 60 min anaerobic filling, 169 min aerobic phase, 3 min settling, 5 min discharging and 3 min idle, as presented in Figure 4.1.

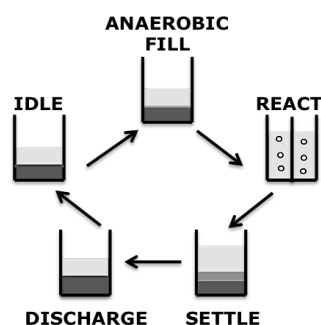
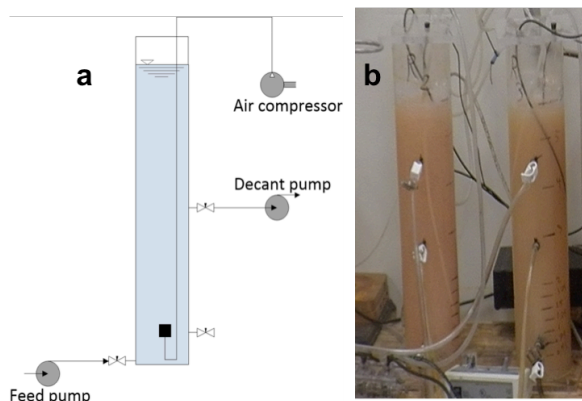


Figure 4-1. Cycle configuration

Porous stones were used as aerators and installed at the bottom of each reactor; the aeration rate was 5 L min<sup>-1</sup>; the dissolved oxygen concentration during the aerobic phase was not controlled, providing not limiting oxygen concentration in the reactors. A set of two peristaltic pumps was used for the feed solution filling and for effluent withdrawn through a port placed at middle height of the reactor (Fig. 4.2). Synthetic wastewater with acetate as the sole carbon source was used as feeding solution and the composition of the medium was the following: NaAc 1000 mg L<sup>-1</sup>, NH<sub>4</sub>Cl 200 mg L<sup>-1</sup>, K<sub>2</sub>HPO<sub>4</sub> 50 mg L<sup>-1</sup>, CaCl<sub>2</sub>·2H<sub>2</sub>O 15 mg L<sup>-1</sup>, MgSO<sub>4</sub>·7H<sub>2</sub>O 12.5 mg L<sup>-1</sup>, FeSO<sub>4</sub> 10 mg L<sup>-1</sup> and trace metals solution 1 mL L<sup>-1</sup>. Trace metals solution composition was the following: FeCl<sub>3</sub>·6H<sub>2</sub>O 1.5 g L<sup>-1</sup>, H<sub>3</sub>BO<sub>3</sub> 0.15 g L<sup>-1</sup>, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.03 g L<sup>-1</sup>, KI 0.03 g L<sup>-1</sup>, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.12 g L<sup>-1</sup>, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.06 g L<sup>-1</sup>, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.12 g L<sup>-1</sup>, CoCl<sub>2</sub>·2H<sub>2</sub>O 0.15 g L<sup>-1</sup> and EDTA 10 g L<sup>-1</sup>. The influent COD concentration was maintained at 850 mg L<sup>-1</sup>, giving rise to an

organic loading rate of  $2.55 \text{ Kg COD m}^{-3} \text{ d}^{-1}$ . The influent  $\text{NH}_4^+\text{-N}$  and  $\text{PO}_4^{3-}\text{-P}$  concentrations were fixed at  $54 \text{ mg N L}^{-1}$  and  $9 \text{ mg P L}^{-1}$  respectively, thus resulting in a COD:N:P ratio of about 100:6:1.



**Figure 4-2. Schematic representation (a) and picture of the SBRs (b)**

Due to operational changes in feeding and stirring strategy, the total operation period consisted of two different stages. During Stage I (from day 1 to day 50), mechanical mixers with a 7.6 cm diameter propeller-type blade were used during the aerobic phase at a constant speed of 180 rpm and the feed solution was introduced in the system from the top of the reactor. During stage II (from day 51 to 206), mechanical mixers were omitted and the feeding strategy was changed from top, to up-flow.

Systems were operated at room temperature ( $20\text{-}22^\circ\text{C}$ ). All reactors were inoculated with conventional fresh activated sludge taken from a full-scale BNR plant, West End Water Pollution Control Centre, Winnipeg, Canada. The inoculated seed sludge was typical flocculent-activated sludge with a fluffy, irregular and loose morphology (Fig. 4-2a), a mean floc size of  $200\text{-}240 \mu\text{m}$  and an SVI of  $108 \text{ mL g}^{-1}$ . The experiment was conducted in duplicate and reactors start-up was conducted in the environmental engineering laboratory of the Department of Civil Engineering of the University of Manitoba.

#### *Analytical methods*

Mixed liquor total and volatile suspended solid (TSS/VSS), effluent total suspended solid (ETSS) and sludge volume index ( $\text{SVI}_{30}$ ) were measured according to standard methods [8]. Soluble COD was measured using Hach Lange digestion vials (High Rate vials  $20\text{-}1500 \text{ mg COD L}^{-1}$ ), while soluble phosphate ( $\text{PO}_4^{3-}\text{-P}$ ), ammonium ( $\text{NH}_4^+\text{-N}$ ), nitrite ( $\text{NO}_2^-\text{-N}$ ) and nitrate ( $\text{NO}_3^-\text{-N}$ ) were measured using an automatic flow injection analyser (Quick Chem 8500, Lachat

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instruments). Particle-size distribution was measured using Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK). For the determination of the average diameter of the granules, only particles with diameter of greater than 200 µm were considered.

#### *Calculatory procedures*

COD and nutrient removal efficiencies were calculated according to the following equations:

$$COD \text{ removal efficiency (\%)} = \frac{COD_{in} - COD_{out}}{COD_{in}} \times 100 \quad [3]$$

$$NH_4 \text{ removal efficiency (\%)} = \frac{NH_4_{in} - NH_4_{out}}{NH_4_{in}} \times 100 \quad [4]$$

$$Total \text{ N removal efficiency (\%)} = \frac{NH_4_{in} - (NH_4 + NO_x)_{out}}{NH_4_{in}} \times 100 \quad [5]$$

$$P \text{ removal efficiency (\%)} = \frac{P_{in} - P_{out}}{P_{in}} \times 100 \quad [6]$$

The average sludge retention time (SRT) was calculated from the biomass concentration in the reactor (dry weight) and the biomass concentration in the effluent, according to Equation 5.

$$Average \text{ SRT} = \frac{V_r X_r}{Q_w X_{out}} \quad [7]$$

Where:

$V_r$  = total working volume of the reactor (L)

$X_r$  = biomass concentration in the reactor (gVSS L<sup>-1</sup>)

$Q_w$  = waste flow rate from the reactor (L d<sup>-1</sup>)

$X_{out}$  = biomass concentration in the effluent (gVSS L<sup>-1</sup>)

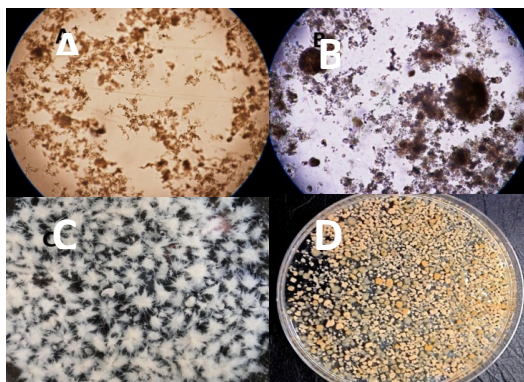
#### **4.1.2 Results and discussion**

##### *Formation and morphology of aerobic granules*



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The reactors were inoculated with suspended growth activated sludge (Fig. 4.3a) from West End Water Pollution Control Center (Winnipeg, MB). The initial cycle consisted of 60 min anaerobic filling, 152 min aerobic phase, 20 min settling, 5 min discharging and 3 min idle; in one month the settling time was shortened gradually to 4 minutes in order to promote the washout of the suspended biomass. First small sludge aggregations appeared three weeks after reactors start-up (Fig. 4.3b), and 4 weeks after first granules could be detected. These granules were not round in shape showing a fluffy structure with filamentous organisms appearing on their surface (Fig. 4.3c), low settling velocity and high SVI ( $> 100 \text{ mL g}^{-1} \text{ TSS}$ ). Filamentous growth has been frequently detected during aerobic granules cultivation; when filamentous bacteria concentration is low they do not cause operational problems and may even stabilize the granule structure acting as a rigid backbone [9]. However, once filamentous become predominant in the reactor they can extend from the granule and bind different particles together, increasing the surface area without a corresponding increase in mass. This leads to a decreasing in the settleability of the sludge and consequently to a biomass washout from the reactor [9].



**Figure 4-3. Sludge morphology at different stages of granulation: microscope images (40X) of seed sludge (a) and first aggregates at day 15 (b), digital camera images of filamentous dominated granules (c) and mature granules at on day 160 (d).**

Due to a filamentous microorganisms overgrowth, 7 weeks after the start-up, the reactor set-up was modified: mechanical mixers were omitted and feeding type was changed from top to up-flow. The irregular flow patterns due to the simultaneous mechanical and hydrodynamic mixing could, in fact, encourage the formation of star-shape pellets. Therefore, the omission of mechanical mixers and the choice to use aeration as the only shear force could provide the appropriate circular flow pattern to encourage the formation of regular and compact granules [10]. Moreover, the feeding strategy was changed from top of the reactor to the bottom, through the settled sludge bed, in order to provide the right amount of feed to the heavier

granules placed on the bottom of the reactor during the anaerobic phase. Granules containing storing bacteria are, in fact, heavier and settle faster than flocculent aggregates, thus reaching easily the bottom of the reactors. Up-flow feeding can enhance granulation by selecting for slow growing bacteria [1]. 20 days after the new reactor set-up filamentous dominated granules started to disintegrate and a new granulation took place, leading to new, smooth, compact granules (Fig. 4.3d). On day 94, sludge in reactor R2 was nearly completely granulized, and the average diameter of granules was around 1.2 mm. After 160 days, mature and compact granules, with average diameter of 1.1 and 1.3 mm, dominated the systems. Monitoring TSS, SVI and SRT in the SBRs, indicated an increase in these three parameters. Biomass concentration on day 201 was 6.5 g VSS L<sup>-1</sup> in R1 and 7.2 g VSS L<sup>-1</sup> in R2 (Fig. 4.4a and 4.4b).

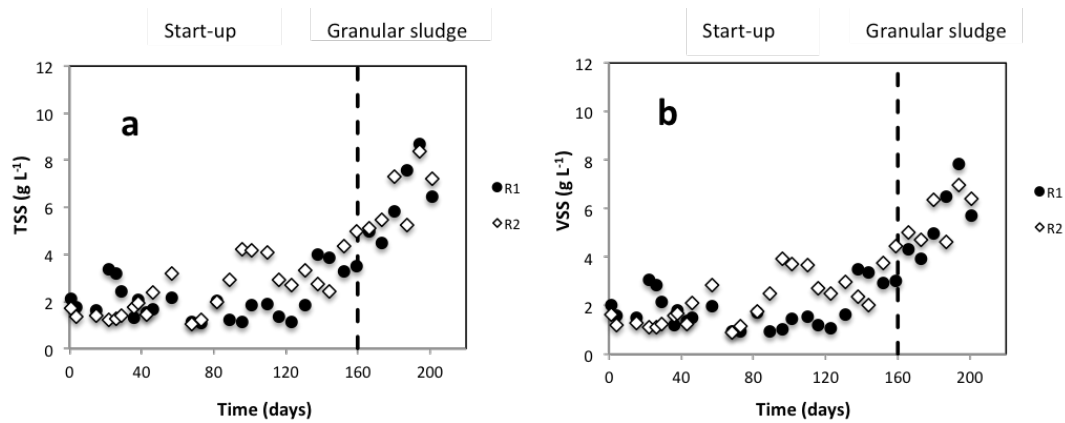


Figure 4-4. Evolution of the biomass TSS (a) and VSS (b) concentration in the reactor.

Sludge volume index is commonly used for the indication of sludge settling ability. The drop of the SVI concurrent with the achievement of a fully granular system, showed a great improvement of sludge settling ability (Fig. 4.5a).

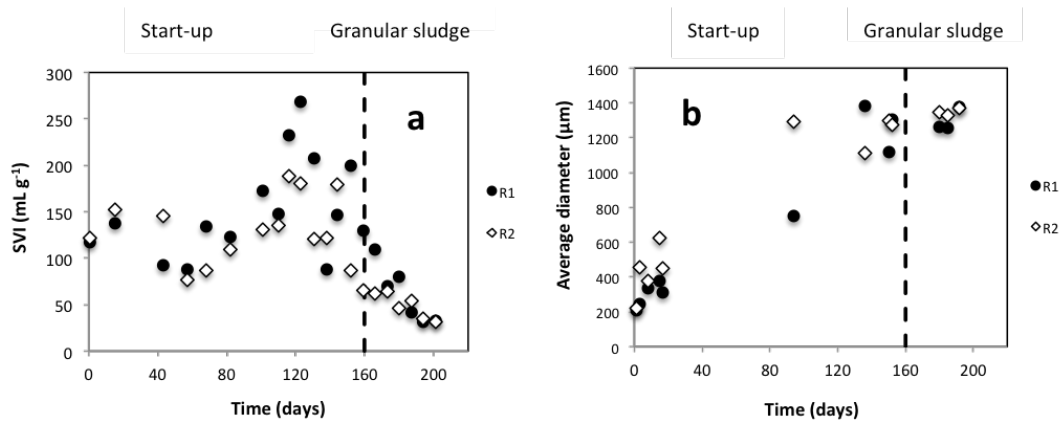


Figure 4-5. Evolution of the sludge volume index (SVI<sub>30</sub>) (a) and average diameter (b) during time.

Simultaneously, the granules size gradually increased in all reactors, and the mean diameter reached a stable value of 1.3 mm at the end of the steady state (Fig. 4.5b).

During the start-up period SRT was fluctuating between 0.4-3 days, depending on the biomass washout in the effluent, which is conventional during aerobic granulation [11-13]. When mature granules dominated the system, SRT increased up to 18 days, thus providing the appropriate conditions to keep microorganisms performing biological nutrient removal in the systems. On the other hand, the high concentration of total solids in the effluent of the reactors ( $>150 \text{ mg L}^{-1}$ ) discloses the need of a further implementation of the system, so to decrease these values within quality effluent limits, which is fundamental for the up scaling of this new technology. A conceivable explanation for this concentration values might be the incomplete COD anaerobic removal, which will be discuss in detail in the following paragraph. An incomplete anaerobic COD removal by slow growing organisms provides, in fact, a readily biodegradable carbon source for filamentous bacteria, which can therefore grow on it, taking advantage of their morphology and outer placement inside the granule. This limited filamentous growth doesn't cause operational problems, as previously mentioned, but the low settling properties of these bacteria cause a significant biomass discharge in the effluent. Therefore, different operational strategies are needed in order to achieve granules dominated by slow growing organisms. The specific characteristics of granular sludge cultivated in R1 and R2 are shown in Table 4.1.

**Table. 4.1. Characteristics of mature granules cultivated in R1 and R2.**

Parameters	Units	R1	R2
TSS	( $\text{g L}^{-1}$ )	6.3	6.4
VSS	( $\text{g L}^{-1}$ )	5.5	5.7
SVI <sub>30</sub>	( $\text{mL g}^{-1}$ )	51.4	46.3
Average diameter	(mm)	1.3	1.35
Average SRT	(days)	9	10

### *Reactor performances*

Aerobic granular sludge was developed using a synthetic feeding medium containing sodium acetate as the only carbon source, with an organic loading rate of  $2.55 \text{ Kg COD m}^{-3} \text{ d}^{-1}$ . The influent COD,  $\text{NH}_4\text{-N}$  and  $\text{PO}_4^{3-}\text{-P}$  concentration was  $850 \text{ mg L}^{-1}$ ,  $54 \text{ mg L}^{-1}$  and  $9 \text{ mg L}^{-1}$  respectively. COD removal efficiency was stable in all three reactors throughout the experiment and it reached more than 90% during the start up and more than 95% during the steady state period. However, according to the typical patterns of acetate, phosphate, and

nitrogen concentrations during a cycle (Fig. 4.6), even after mature granules appearance, anaerobic COD removal was incomplete. As previously mentioned, an incomplete anaerobic COD storage by slow growing organisms provides a readily biodegradable carbon source for conventional fast growing heterotrophs present in the outer layer of the granule. The growth of conventional suspended heterotrophs enhances biomass concentration in the effluent, since the settling properties of flocculent biomass are not good enough to be retained in the system, and it also limits oxygen availability for nitrifiers. In fact, ammonium uptake rate increases just after COD depletion; as long as COD is available during the aerobic phase for the external heterotrophs, nitrifiers are oxygen limited (Fig. 4.6). Although the total COD removal efficiency reached more than 95% in all reactors, the incomplete anaerobic COD storage reveals relevant operational dysfunctions, which need to be solved in order to enhance the selection for slow growing organisms and consequently improve granule long-term stability and reduce biomass discharge in the effluent.

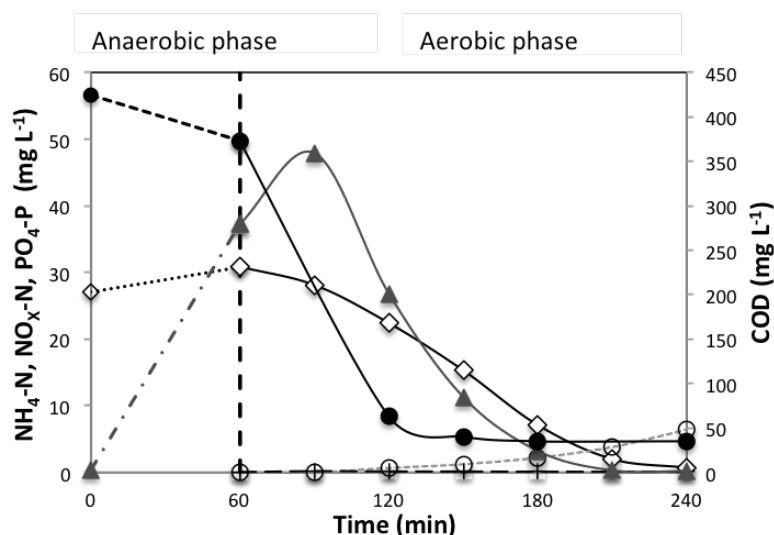


Figure 4-6. Concentration patterns of COD (●), ammonium (◇), nitrite (○), nitrate (+) and phosphate (▲), during a cycle.

During the start-up, nitrogen removal was unstable because of a washout of slow growing nitrifying biomass due to the short SRT, with ammonium concentration in the effluent ranging between 8 and 20 mg L<sup>-1</sup> (Fig. 4.7a). Simultaneously with the dominance of mature granules and the increase in the SRT, the concentration of ammonium in the effluent decreased to zero and the ammonium removal efficiency reached up to 100% in all reactors (Fig. 4.8d). Small nitrate concentration in the effluent (Fig. 4.7c) reveals the occurrence of a simultaneous nitrification/denitrification (SND) (or nitrification/denitrification) process. SND takes place within aerobic granules and is directly related to the microbial species distribution and cycle set-up.

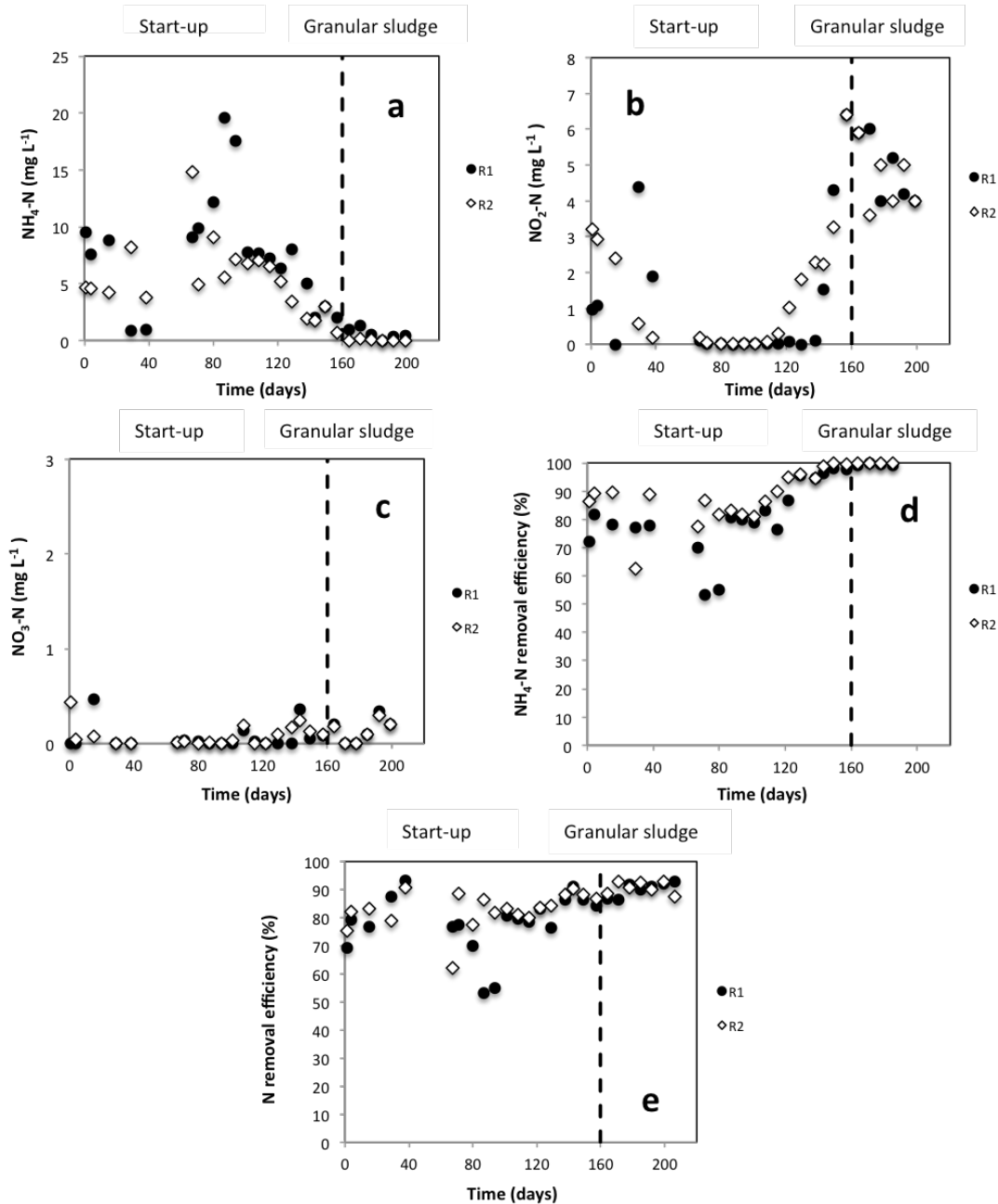


Figure 4-7. Evolution of nitrogen compounds concentrations  $\text{NH}_4\text{-N}$  (a),  $\text{NO}_2\text{-N}$  (b),  $\text{NO}_3\text{-N}$  (c) in the effluent of the SBRs and  $\text{NH}_4\text{-N}$  (d) and total N (e) removal efficiency during time.

During the feast period (when VFA are available), according to the movement of solutes along a concentration gradient, organic substrate penetrates into the granules to be there anaerobically stored as PHA. During the famine period (no external acetate available), when oxygen is present for autotrophic organisms, ammonium is converted to nitrate. Nitrate can penetrate to the inner layers of the granule, where the cell-internally stored substrate of DPAO or DGAO serves as electron donor for denitrification [14]. However, according to the cycle profile, starting from minute 150, when readily biodegradable COD has been totally removed, a nitrite accumulation occurs in the system, giving rise to high effluent nitrite concentrations.

The concomitance of nitrite accumulation and COD depletion suggests that conventional denitrifiers, instead of denitrifying glycogen accumulating organisms (DGAO) and denitrifying poly-phosphate accumulating organisms (DPAO), might be responsible for denitrification. Regarding total nitrogen removal efficiency, an average removal efficiency of 88% was achieved in all reactors.

According to van Loosdrecht et al. [15], a suitable parameter to enhance granule stability is the growth rate of the constituent microorganisms, and so, selecting for slow growing organisms, instead of ordinary heterotrophic organisms is advantageous to increase granule stability and removal efficiencies. Moreover, the activity of ordinary denitrifiers, which would reduce nitrate, using COD as electron donor, results in less COD available for PAO growth. Considering all this factors, different operational strategies are then needed so to enrich granules culture with slow growing organisms.

Biological phosphorus removal efficiency was low during the initial phase of the experiment, ranging from 40 to 70% (fig. 4.8b) and it was mainly due to cell growth.

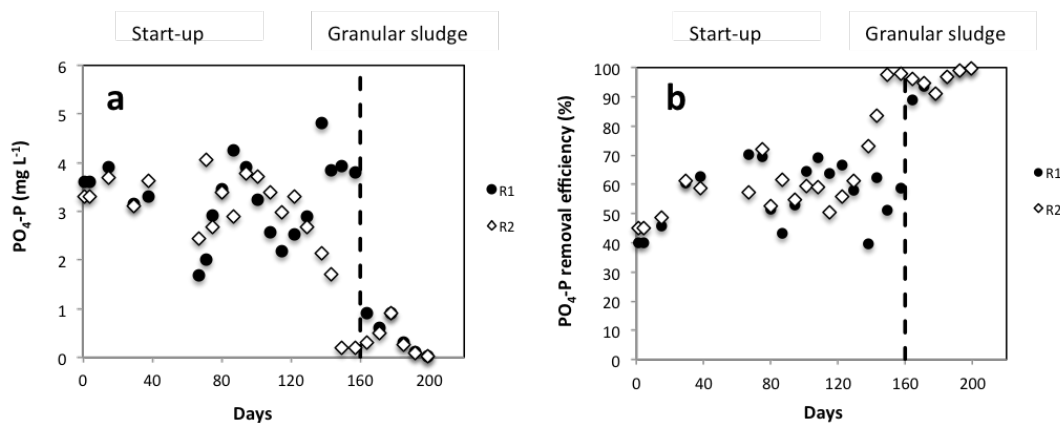


Figure 4-8. Evolution of phosphorous removal efficiency and phosphorous concentration in the effluent during time.

After granules maturation and PAOs enrichment, anaerobic phosphorus release was observed. Once again, the release of phosphorus during the first 30 min of the aerobic phase (Fig. 4.6) indicates that the inner layers of the granule are still anoxic, due to the presence of high COD concentration in the bulk, which prevents oxygen diffusion inside the granule. After almost 160 days from the beginning of the experiment, phosphorus concentration in the effluent decreased to almost zero (Fig. 4.8a) and phosphorus removal started to increase, reaching up to 95% in all reactors. Final average nutrient removal efficiencies of mature granules are reported in Table 4.2. The similarities in the reactors performances demonstrated the compliance of the achieved results.

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**Table. 4.2. Nutrient removal efficiencies of mature granules cultivated in R1 and R2.**

Parameters	Units	R1	R2
NH <sub>4</sub> -N removal efficiency	(%)	99	100
TN removal efficiency	(%)	91	92
P removal efficiency	(%)	97	97
COD removal efficiency	(%)	96	96

### 4.1.3 Conclusions

Granulation of activated sludge was achieved in three laboratory-scale SBRs fed with a synthetic medium containing sodium acetate as the only carbon source. Mature granules showed a smooth and round surface, with a mean diameter of 1.3 mm and a final average SVI<sub>30</sub> of 48 mL gTSS<sup>-1</sup>. However, a filamentous bacteria overgrowth occurred during the process of granulation. It was found that a change in the feeding strategy, from top to up-flow, and the exclusion of mechanical mixers during the aerobic phase, was beneficial to the granulation process. Up-flow feeding can, in fact, enhance granulation by selecting for heavier aggregates, characterized by good settling properties. Results showed that simultaneous COD, nitrogen and phosphorous removal was achieved in all reactors. Despite this, the system needs to be further optimized. Total COD removal efficiency was stable throughout the experiment and it reached up to 95%. N and P removal performances were unstable during the first phase of granulation, but they increased once mature granules become dominant in the system, reaching up to 89 and 96%, respectively.

On the other hand, anaerobic COD storage was incomplete, leading to the availability a of readily biodegradable carbon source during the aerobic phase. The incomplete anaerobic COD storage by slow growing organisms caused the main instabilities of the system, which can be summarized as follows:

- Suspended biomass growth on easy biodegradable substrate during the aerobic phase, responsible for the high solids concentration in the effluent and for the oxygen mass transfer limitation;
- Low enrichment of slow growing organisms (PAO/DPAO and GAO/DGAO), which results in a conventional SND process, instead of a process of simultaneous nitrification and denitrification and phosphorus removal (SNDPR). Therefore, conventional denitrifiers, instead of DPAO/DGAO, were responsible for most of the denitrification in this system.

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Since granule stability is dependent on the growth rate of the microorganisms, selecting for slow growing bacteria is crucial to improve aerobic granular sludge stability and nutrient removal performances. Results showed that PAO enrichment achieved in this study is not sufficient to remove completely the COD during the anaerobic phase, and it is mainly due to the competition between PAO and ordinary heterotrophic organisms for the organic substrate under anoxic conditions. Different operational strategies are then needed so to enrich granules culture with slow growing organisms.



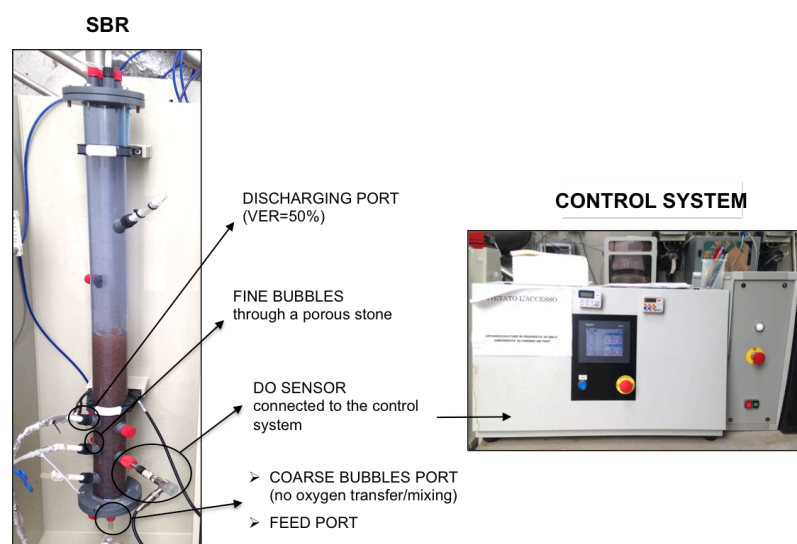
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## Start-up of an aerobic granular sequencing batch reactor

### 4.1.4 Materials and methods

#### *Experimental set-up*

Prior aerobic cultivation experiment disclosed several operational dysfunctions; therefore a new reactor set-up was adopted, in order to apply the most favourable conditions previously determined and to enhance the selective pressure due to the height to diameter ratio (H/D). Besides, the reactor was fed with a mixture of synthetic medium and domestic sewage, in order to investigate aerobic granule formation on a complex, real influent. Aerobic granules cultivation was run in a laboratory-scale SBR, with an internal diameter of 8 cm, total working volume of 3 litres and volume exchange ratio of 50%, as sketched in Figure 4.9.



**Figure 4-9. Reactor set-up.**

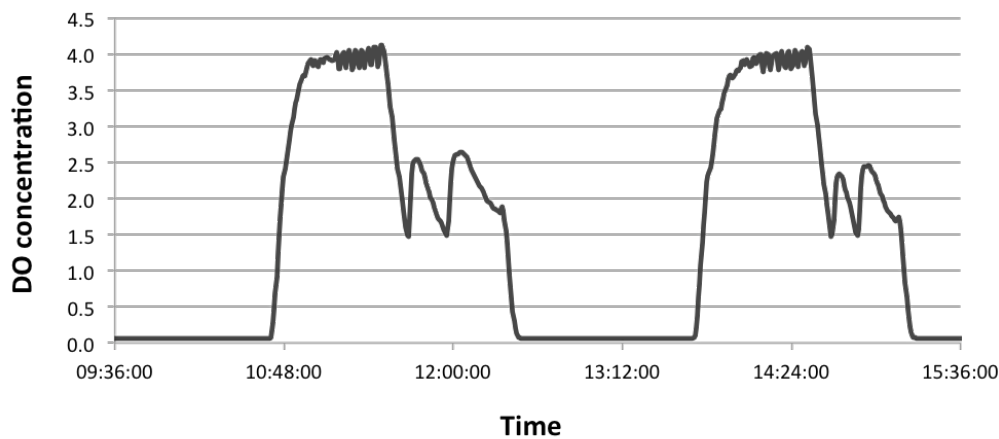
The reactor was operating sequentially in 3-hour cycle comprising 60 minutes of anaerobic feeding, 110 minutes of aeration, 2 minutes settling and 3 min discharging. The cycle length was shorter compared to the previous experiment, so to increase the number of cycles per day and the selective pressure due to an alternating feast/famine regime. Due to operational changes in DO concentration inside the reactor, the total operation period consisted of three different stages (Tab. 4.3). During Stage I (from day 1 to day 176) aeration period included two different phases: 60 minutes with DO concentration set between 3.9 and 4 mg L<sup>-1</sup> (Aeration 1) and 50 minutes with DO concentration set between 1.5 and 2 mg L<sup>-1</sup> (Aeration 2). During Stage II (from day 176 to day 239), Aeration 1 was extended to 90 minutes, with DO concentration set

to 9 mg L<sup>-1</sup>, and Aeration 2 was shorten to 20 minutes with DO concentration set between 1.5 and 2 mg L<sup>-1</sup>. During Stage III (from day 239 to day 280), Aeration 1 was shorten to 60 minutes and DO concentration decreased to 6 mg L<sup>-1</sup>, while parameters of Aeration 2 were the same as Stage I.

**Table. 4.3. Characteristics of the three operational stages of aeration phase.**

	Aeration 1		Aeration 2	
	Length (min)	DO (mg L <sup>-1</sup> )	Length (min)	DO (mg L <sup>-1</sup> )
Stage I	60	3.9-4.0	50	1.5-2.0
Stage II	90	9	20	1.5-2.0
Stage III	60	5.9-6.0	50	1.5-2.0

Dissolved oxygen concentration profile during Stage I is shown in Figure 4.10.



**Figure 4-10. DO concentration profile during time.**

DO concentration inside the reactor was regulated through a DO sensor connected to a control system. Fine bubbles supplied by a porous stone, placed at the bottom of the reactor, were used to increase DO concentration to the higher set point limit, while decreasing the oxygen saturation at lower concentrations occurred by means of coarse bubbles introduced at the bottom of the reactor through the port used for filling. Moreover, coarse bubbles were also used to maintain a constant mixing when no oxygen transfer was needed.

A set of two peristaltic pumps was used to introduce in plug-flow conditions the concentrated feeding solution and the domestic sewage through the settled sludge bed. This feeding strategy provides a substrate concentration in the lowest part of the settled bed equivalent to the influent substrate concentration. The composition of the synthetic medium was the

following: 8.6 g L<sup>-1</sup> NaAc, 2.38 g L<sup>-1</sup> (NH<sub>4</sub>)HCO<sub>3</sub>, 1.965 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>. Each cycle 72 mL of the synthetic medium was added to the reactor together with 1500 mL of domestic influent, corresponding to a synthetic/real wastewater ratio of 5%. The organic loading rate and the influent COD, NH<sub>4</sub>-N and PO<sub>4</sub>-P concentrations due to the synthetic medium were respectively 1.6 Kg m<sup>-3</sup> d<sup>-1</sup>, 320 mg L<sup>-1</sup>, 20 mg L<sup>-1</sup>, and 20 mg L<sup>-1</sup>. The synthetic medium was provided in order to support the microbial growth since the domestic sewage nutrient concentrations are low and they are also affected by seasonal fluctuation; the monthly average concentrations registered during the experiment are reported in Table 4.4.

**Table. 4.4. Monthly average domestic sewage COD, N and P concentration.**

Month	COD	NH <sub>4</sub> -N	PO <sub>4</sub> -P
August	84.0	18.2	1.5
September	48.2	15.9	1.3
October	58.9	23.1	1.4
November	37.8	13.6	1.4
December	30.4	17.5	1.5
January	67.7	15.8	1.5
February	39.7	13.5	1.7
March	33.2	13.2	1.7
April	42.5	15.1	2.2
May	25.0	19.4	1.5

During the first 40 days of the start-up period, allylthiourea (ATU) was dosed to reach a final concentration in the reactor of 100 mg L<sup>-1</sup>, so to prevent nitrification process and the presence of nitrate during the feeding phase, which would impede the development of PAO population [16]. The presence of nitrate, in fact, would trigger the activity of denitrifiers, resulting in less COD available for PAO growth and would impede anaerobic conditions necessary for COD uptake by PAO. No pH or temperature control was operated, in order to reproduce real operating conditions (although both parameters were constantly monitored through a pH and a temperature probe). Therefore, the reactor and microbial populations were subjected to seasonal fluctuation of temperature (from 10 to 28°C) and operating fluctuation of pH (from 7.7 during the feeding phase, to 8.5 during aeration phase). The reactor was inoculated with conventional floccular activated sludge, collected from the full-scale WWTP of San Colombano, Florence, Italy, and was operated for 280 days (from August 2014 to May 2015).

#### *Analytical methods*

Mixed liquor total and volatile suspended solid (TSS/VSS), effluent total and volatile suspended solid (ETSS/EVSS) and sludge volume index (SVI<sub>5</sub> and SVI<sub>30</sub>) were measured according to

standard methods [8]. Soluble COD, phosphate (PO<sub>4</sub>-P), ammonium (NH<sub>4</sub>-N), nitrite (NO<sub>2</sub>-N) and nitrate (NO<sub>3</sub>-N) were measured using Hach Lange kit LCK 614, LCK 348, LCK 303, LCK 341 and LCK 339 respectively. The settled granular sludge bed height was determined at the end of the discharging phase, according to the graduated scale present on the external side of the reactor. Average diameter of granules was roughly determined by sieving the granules using 0.8, 1 and 1.5 mm sieves.

#### *Calculatory procedures*

COD and nutrient removal efficiencies were calculated according to equations [3-6]; the average sludge retention time (SRT) was calculated according to equation [7].

The oxygen penetration depth was calculated according to the following equation [17]:

$$\delta_{pf} = \sqrt{\frac{2D \cdot C_{si}}{q_{O_2}^{max} \cdot c_{xf}}} \quad [8]$$

$\delta_{pf}$  = oxygen penetration depth (m)

D = oxygen diffusion coefficient (m<sup>2</sup> s<sup>-1</sup>)

C<sub>si</sub> = oxygen concentration in the biofilm interphase (mol O<sub>2</sub> L<sup>-1</sup>)

q<sub>O<sub>2</sub></sub><sup>max</sup> = maximal oxygen specific uptake rate (mol O<sub>2</sub> g<sup>-1</sup> VSS sec<sup>-1</sup>)

c<sub>xf</sub> = biomass in the biofilm (gVSS L<sup>-1</sup>)

### **4.1.5 Results and discussion**

#### *Formation and morphology of aerobic granules*

The reactor was started up at 28°C with no pH and temperature control. The seed sludge was conventional flocculent activated sludge, characterized by a fluffy and loose morphology (Fig. 4.11a) and an SVI of about 100 mL g<sup>-1</sup>. The morphological evolution of the activated seed sludge at different granulation stages is shown in Figure 4.11.

First small aggregations (Fig. 4.11b) were detected 2 weeks after inoculation, and first small granules (Fig. 4.11c) appeared after 30 days. At this stage granules exhibited an irregular, fluffy outer structure due to filamentous bacteria extruding from granule surface. 40 days after inoculation, ATU was removed from the feed in order to allow nitrifiers development. During the next stages, granules became bigger and denser and about 50 days after the start-up

mature granules, with a smooth-outer morphology, dominated the system (Fig. 4.11e). As the granules matured, the mean biomass size gradually increased and the average diameter reached a stable value between 1.3 and 1.5 mm (Fig. 4.11f); at the same time granules displayed a smooth outer morphology that did not change noticeably during the rest of the experiment (Fig. 4.11h).

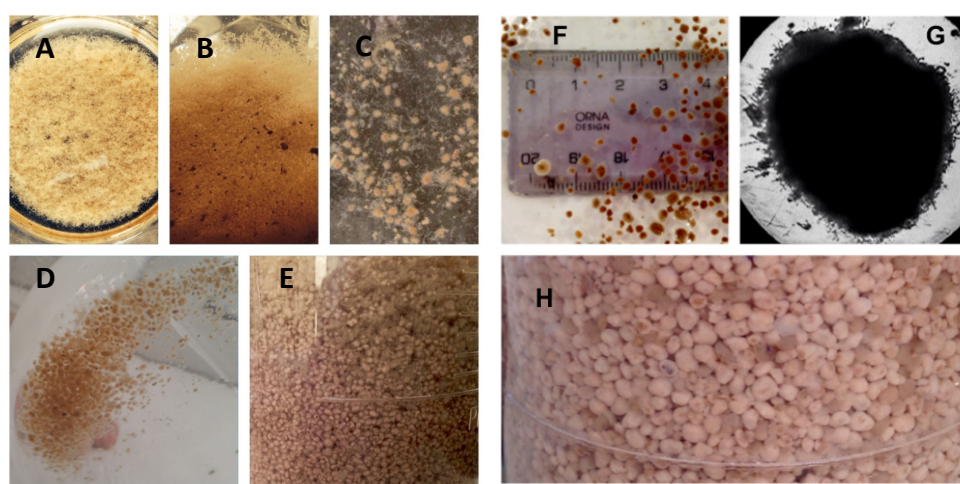


Figure 4-11. Evolution of the sludge in the reactor (a, b, c, d and e), mature granules diameter (f), microscope image (10X) (g) and morphology and on day 190 (h).

Two months after inoculation, first alginate-like granules were detected in the system (Fig. 4.12a). Alginate is an exopolysaccharide, with unique gel-forming property [18], secreted in nature by only two microbial genera: *Pseudomonas* and *Acetobacter* [19].

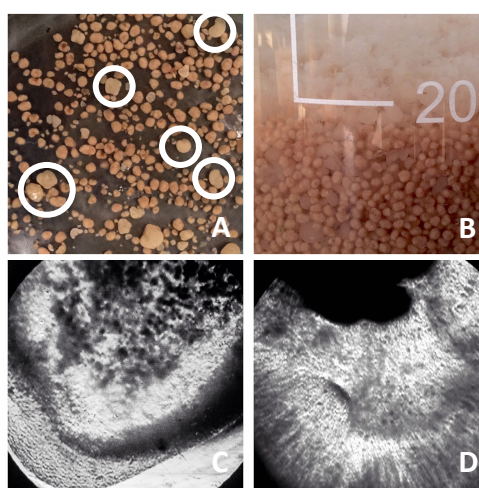


Figure 4-12. Alginate-like granules appearance (a), stratification conventional/alginate-like granules after settling (b) and microscope images (10X) of the external layer of the granule (c, d).

The ability to secrete different kind of exopolysaccharides is widespread among microorganisms because it serves functions such as resistance against floc-water loss, adherence to surfaces, promotion of microbial aggregation (e.g., biofilms), and protection against specific and non-specific host immunity. Alginate-like granules developed during a stage of the start-up when anaerobic COD storage was still incomplete, thus providing a readily biodegradable source of organic carbon during the aerobic phase. These granules, showed lower settling velocity ( $42 \pm 5 \text{ m h}^{-1}$ ) compared to conventional aerobic granules ( $101 \pm 15 \text{ m h}^{-1}$ ), causing a stratification of the two granular types after the settling phase (Fig. 4.12b). However, once all the available COD started to be fully stored under anaerobic conditions, alginate-like granules gradually broke up and disappeared from the system, presumably because of the lack of a carbonaceous source available during the aerobic stage.

The reactor was operated for 280 days; the first 220 were used to stabilise the conversion processes. The evolution of the biomass total and volatile suspended solids concentration in the reactor is shown in Figure 4.13. As mature granules dominated the system, an increase in total and volatile suspended solids concentration occurred. After the start-up period, on day 235, biomass concentrations reached up to  $27.4 \text{ gTSS L}^{-1}$  and  $12 \text{ gVSS L}^{-1}$ . The decrease in the ratio between volatile and total suspended solids during time is a consequence of the enhanced biological phosphorus removal process (EBPR), which leads to the accumulation of inorganic P in the form of precipitates in the inner core of the granules.

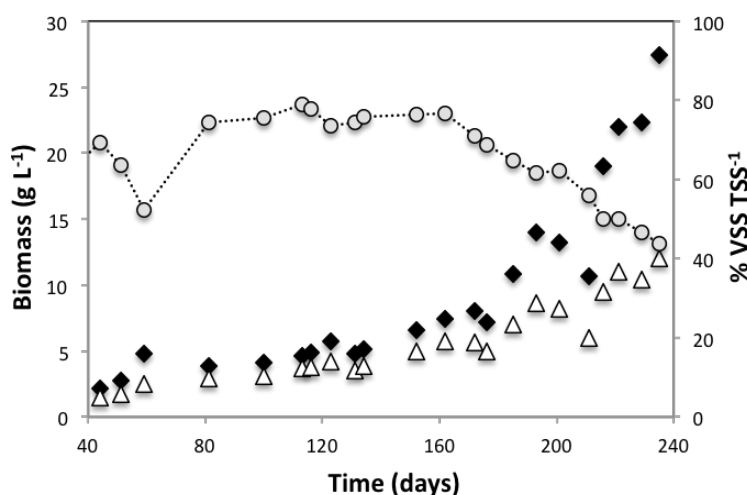


Figure 4-13. Evolution of TSS (◆) and VSS (◻) concentration in the reactor and percentage of their ratio (●).

At the same time, the increase in granule density from  $38$  to  $90 \text{ gVS L}_{\text{biomass}}^{-1}$  (Fig. 4.14) resulted in a low  $\text{SVI}_{30}$ , which, at the end of the start-up period, fluctuated between  $27$  and  $11 \text{ mL gTSS}^{-1}$ .

This value is very low compared to that of conventional activated sludge (Fig. 4.15), which is generally comprised in a range varying between 100 and 150 mL gTSS<sup>-1</sup> [16], and underlines the very good settling properties of aerobic granules achieved. Only SVI<sub>30</sub> values are reported, since they are comparable to those obtained after 5 minutes settling (SVI<sub>5</sub>) (SVI<sub>30</sub>/SVI<sub>5</sub> < 5%). The SVI<sub>5</sub> to SVI<sub>30</sub> ratio is actually an efficient tool to indicate the granulation stage of a system, and for fully granulated systems it is reported to be around 1 [20].

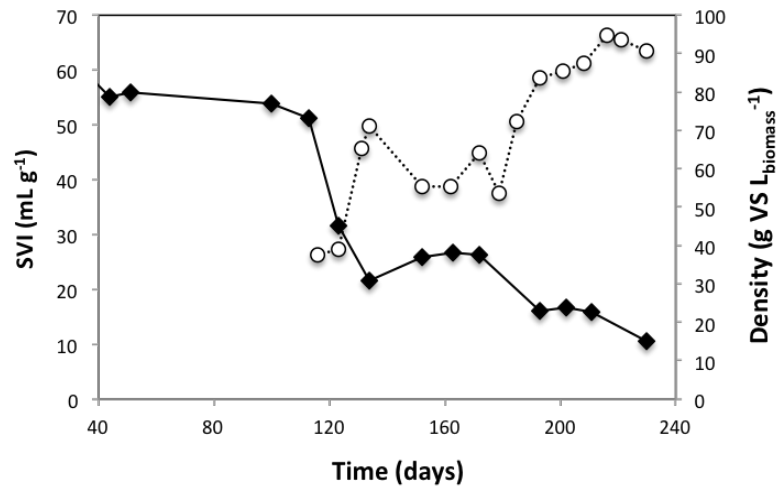


Figure 4-14. Evolution of the sludge volume index (SVI<sub>30</sub>) (◆) and granules density (○) during time.

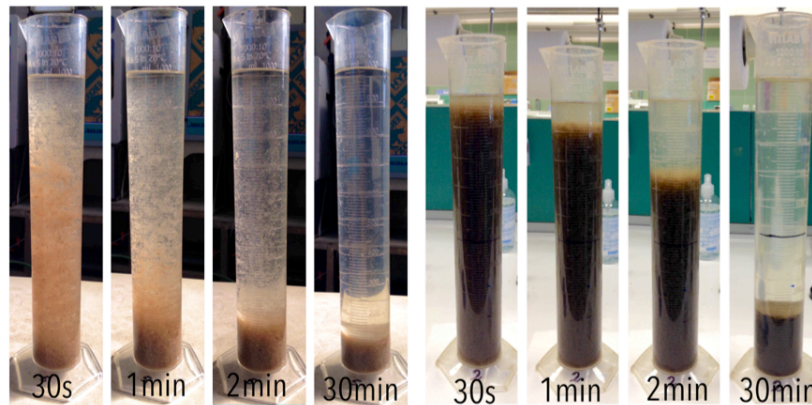


Figure 4-15. Comparison between aerobic granules and activated sludge settling properties.

The average SRT, determined by the biomass influent and the biomass washout in the effluent of the reactor, reached 73 days on day 230. The SRT values achieved comply with those reported for other studies; for example de Kreuk and van Loosdrecht [21] reported SRT values between 40 and 71 days. Contrary to previously described preliminary study, biomass concentration in the effluent during the steady-state period drop to 48 mgTSS L<sup>-1</sup>, a value

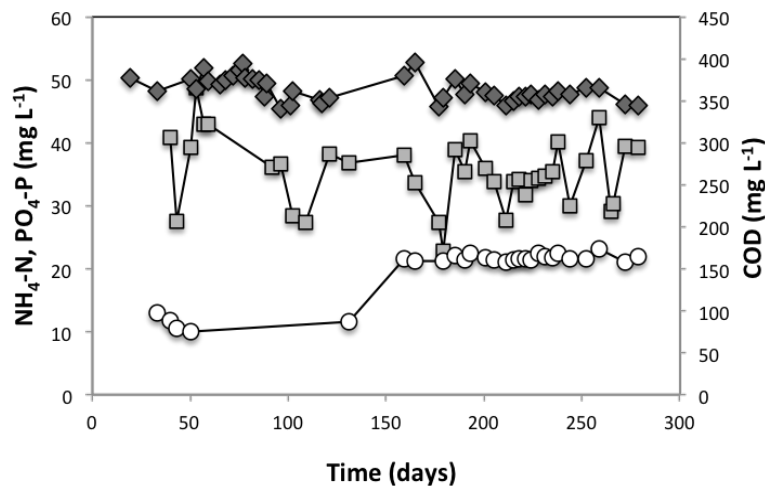
which is very close to Italian quality effluent limits ( $35 \text{ mg TSS L}^{-1}$ ). The average characteristics of granular sludge during the steady-state period are shown in Table 4.5.

**Table. 4.5. Steady-state characteristics of mature granules.**

Parameters	Units	R1
TSS	( $\text{g L}^{-1}$ )	24
VSS	( $\text{g L}^{-1}$ )	11
VSS/TSS	(%)	47
SVI <sub>30</sub>	( $\text{mL g}^{-1}$ )	15
Average diameter	(mm)	1.5
Average SRT	(days)	70

### Reactor performances

The reactor was started-up with dissolved oxygen concentration set between 4 and  $3.9 \text{ mg L}^{-1}$  (Stage I), in order to run a cost-effective system in terms of aeration costs, and without pH and temperature control, so to reproduce real operating conditions. Both the domestic sewage and the synthetic medium were supplied in plug-flow conditions. This feeding strategy, which provides a substrate concentration in the lowest part of the settled bed equivalent to the influent substrate concentration, was indicated by our previous experiment as the more efficient to select for slow growing organisms, able to enhance granules stability under low oxygen concentrations [21]. The influent COD,  $\text{NH}_4\text{-N}$  and  $\text{PO}_4\text{-P}$  concentration due to the synthetic medium was 320, 20 and  $20 \text{ mg L}^{-1}$  respectively, while total influent COD,  $\text{NH}_4\text{-N}$  and  $\text{PO}_4\text{-P}$  concentration were higher due to the COD,  $\text{NH}_4\text{-N}$  and  $\text{PO}_4\text{-P}$  contained in the domestic sewage as depicted in Figure 4.16.



**Figure 4-16. COD (◆),  $\text{NH}_4\text{-N}$  (○) and  $\text{PO}_4\text{-P}$  (■) influent total concentration.**



To avoid nitrification process and ensure anaerobic conditions during the feeding phase, ATU was dosed during the first 40 days. Regarding reactor removal performances, a stable process was not obtained during the first 200 days of operation. In fact, even though a 90% COD removal efficiency was obtained starting from day 57 (Fig. 4.17a), the anaerobic storage of COD resulted incomplete throughout the first 175 days (Fig. 4.17b), thus indicating that C-removal was run by conventional heterotrophs instead of slow growing organisms.

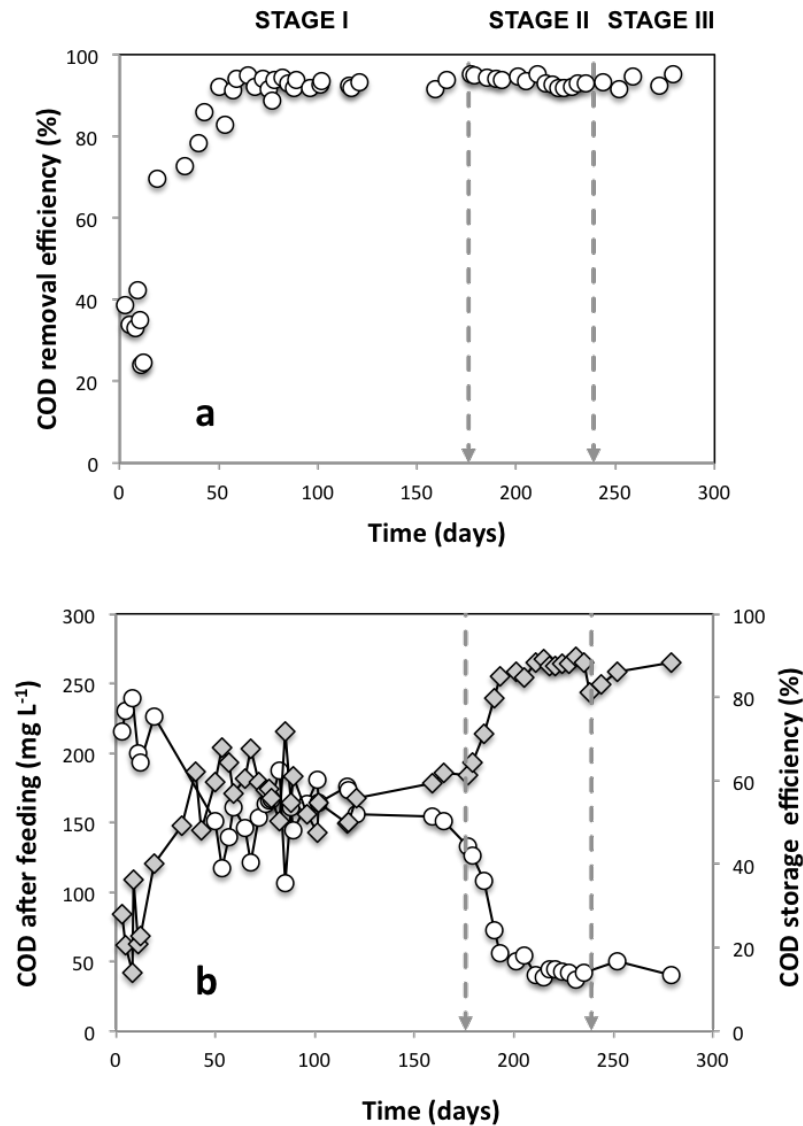


Figure 4-17. COD removal efficiency (a), evolution of COD concentration at the end of the anaerobic feeding phase (○) and evolution of the anaerobic removal efficiency (◆) (b).

The presence of a readily biodegradable source of carbon during the aerobic phase reveals some operational dysfunctions, which resulted in a small enrichment of slow growing organisms (e.g., PAO and GAO), and can also lead to a filamentous overgrowth. Specifically, PAO are able to uptake and store VFA intracellularly as PHA under anaerobic conditions, while

under aerobic (or anoxic) conditions, the stored PHA is used for cell growth, polyphosphate synthesis and glycogen restore. Acetate storage is dependent on the availability of poly-P, whose storage results to be faster under aerobic conditions rather than anoxic [22]. At higher oxygen concentration the penetration depth within the granules increases and more PAO will be able to store phosphate aerobically. The presence of COD during the aerobic phase leads to a high oxygen consumption by the heterotrophs placed in the external layer of the granule and thus to a lower oxygen penetration towards the inner part of the granule. Therefore, in order to increase oxygen penetration depth and allow also PAO, residing more internally, to store phosphate aerobically, on day 176, DO concentration during Aeration 1 was increased from 4 to 9 mg L<sup>-1</sup> and the phase was extended to 90 minutes (Stage II). Starting from day 177, the concentration of COD detected at the end of the anaerobic feeding phase started to decrease (Fig. 4.18) and on day 193 the anaerobic COD removal efficiency reached up to 85%. During the rest of the experiment the anaerobic COD storage efficiency remained stable, reaching up to 90%, despite further decrease in DO concentration.

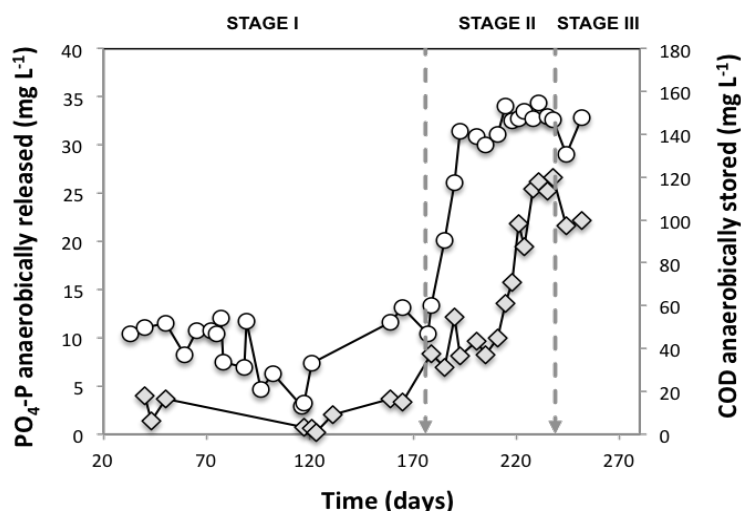


Figure 4-18. Evolution of the anaerobic COD uptake (o) and PO<sub>4</sub>-P release in the bulk (♦).

According to PAO metabolism, COD and P-removal are strictly related [23, 24]. In fact, during the first 175 days of operation the average phosphate release into the bulk liquid during the anaerobic feeding phase was 2 mg L<sup>-1</sup>, while during the period from 200 to 260 days, when anaerobic COD storage was higher than 85%, the average phosphate release was 19 mg L<sup>-1</sup> (Fig. 4.18). It resulted in a ratio between anaerobic P-released and C-uptake of 0.19 P-mol C-mol<sup>-1</sup>. This ratio may suggest if PAO or GAO are the dominant population in the system, starting from the assumption that PAO are the only bacteria able to release phosphate during anaerobic VFA storage. For this reason, high P-released/C-uptake values (>0.5 P-mol C-mol<sup>-1</sup>) indicate PAO

prevalence, whereas low values ( $<0.25$  P-mol C-mol<sup>-1</sup>) indicate GAO prevalence [25-27]. Accordingly, the ratio measured in this study might indicate a GAO-dominated system. On the other hand, considering the operating conditions inside the reactor in terms of temperature (average value lower than 20°C), and pH (7.7-7.9 during the anaerobic feeding phase), GAO should be outcompeted by PAO [28-31]. A possible explanation is the fact that the reliability of the estimations based on the P-released/C-uptake ratio is actually subject of controversy. In fact, many studies demonstrated that this ratio can be influenced by many other factors (carbon source, pH, poly-P content, different clades of PAO) than just the presence of GAO [25, 30, 32, 33], and a very wide range of values have been reported [34]. Therefore, as no molecular analysis was performed to identify the main microbial populations, and the increase in P-release clearly shows the presence of PAO, the issue still remains open and further analysis is needed in order to quantify the two populations of slow growing organisms. A fraction of both populations, called denitrifying PAO (DPAO) and denitrifying GAO (DGAO), can oxidize the internally stored COD using nitrate and/or nitrite instead of oxygen as electron acceptor to achieve P removal (DPAO) and glycogen replenishment (DGAO). Since an anoxic space is required for denitrification, on day 239, DO concentration during aeration 1 was reduced from 9 to 6 mg L<sup>-1</sup> and the phase shortened to 60 minutes. Aim of this operational change was enhancing denitrification process by decreasing the oxygen penetration depth (i.e. increasing the anoxic fraction of the granule volume) and investigating granules performances at lower DO so to reduce operational costs related to aeration. P-release slightly decreased, but total P-removal was not affected, reaching up to 96%.

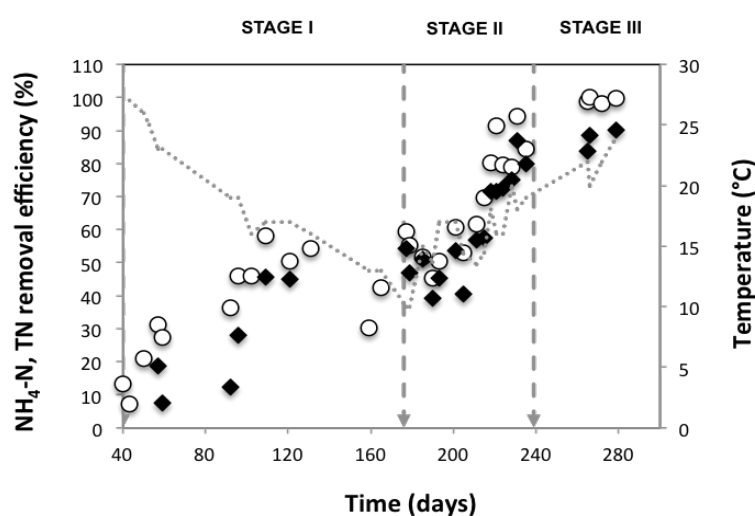


Figure 4-19. Evolution of NH<sub>4</sub>-N (○) and TN (◆) removal efficiency during time.

After ATU removal, nitrogen compounds concentrations were measured; NH<sub>4</sub>-N removal was

highly fluctuating during the first 175 days, with an average efficiency of 36% and  $\text{NH}_4\text{-N}$  concentration in the effluent of  $23 \text{ mg L}^{-1}$  (Fig. 4.19). The main reason is to be found in a combined effect of washout of nitrifying biomass growing on filamentous sludge and seasonal temperature fluctuation. In fact, during the start-up period, when fluffy sludge was still present inside the reactor, nitrifiers may prefer to attach on filamentous sludge, rather than granules, because of the greater available surface and higher DO concentration (due to a lower oxygen mass transfer limitation). Fluffy sludge retention time is short (3 d) because of the reduced settling time; therefore nitrifiers growing on suspended sludge are easily washed out from the system.

This assumption was confirmed by a batch test, aimed at comparing the specific ammonia uptake rate (sAUR) of suspended sludge withdrawn with the effluent, with sAUR measured for aerobic granules. Batch test was performed at  $20^\circ\text{C}$ , under fully aerobic conditions. Results showed an ammonium uptake rate of  $32 \text{ mg NH}_4\text{-N g}^{-1} \text{ VSS h}^{-1}$  for suspended sludge, and  $2.9 \text{ mg NH}_4\text{-N g}^{-1} \text{ VSS h}^{-1}$  for granular biomass. The difference between sAUR values is a clear demonstration that most of the nitrifying biomass is not retained in the system because of its location on fluffy sludge. After the system was completely dominated by granular sludge and no more suspended sludge was available, nitrifiers started to attach on granules surface, increasing their concentration in the system. Granules are characterized by a long SRT (around 60 d), which allows nitrifiers' retention and development inside the reactor, leading to an overall increase in nitrogen removal.

Regarding temperature, according to Arrhenius law (Eq. 9), the rate of biological conversion processes is strictly temperature-dependent:

$$k(T) = k(20) \theta^{(T-20)} \quad [9]$$

Where:

$K(T)$  = conversion rate ( $\text{h}^{-1}$ ) at temperature  $T(^{\circ}\text{C})$

$\theta$  = constant experimentally determined (1.12 for nitrifiers; 1.07 for heterotrophs) [11].

Consequently, nitrifiers and heterotrophs kinetic results to be halved every 6.12 and  $10.25^\circ\text{C}$  respectively, with nitrifiers resulting the more temperature-sensitive microorganisms.

From day 176, a higher oxygen concentration in the bulk and the gradual disappearance of residual filamentous suspended sludge (with the consequent growth of nitrifiers on granules) resulted in a higher nitrification capacity, with an average  $\text{NH}_4\text{-N}$  removal efficiency of 56% and  $\text{NH}_4\text{-N}$  concentration in the effluent of  $14.5 \text{ mg L}^{-1}$  after 40 days of operation at oxygen

saturation. Furthermore, from day 218, the increase of temperature above 18°C, induced a rapid increase in the nitrification rate and from day 265 no ammonium could be detected in the effluent, reaching a  $\text{NH}_4\text{-N}$  removal efficiency of 100%. The evolution of nitrogen compounds concentrations in the effluent is shown in Figure 4.20.

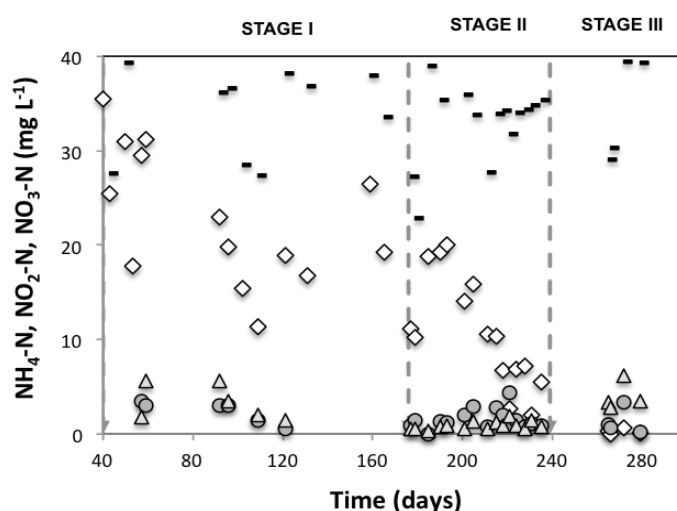


Figure 4-20. Evolution of nitrogen compounds concentrations:  $\text{NH}_4\text{-N}$  in the influent (-) and  $\text{NH}_4\text{-N}$  (◇),  $\text{NO}_2\text{-N}$  (●),  $\text{NO}_3\text{-N}$  (▲) in the effluent.

Low nitrite and nitrate concentration in the effluent reveals the occurrence of simultaneous nitrification/denitrification (SND) (or nitrification/denitrification) process. The increase in the total nitrogen removal efficiency from day 176 onwards, suggests also that denitrification process was carried out by DPAO-DGAO instead of conventional denitrifiers. In fact, the absence of an available carbon source during the aerobic phase, when ammonium is converted to nitrate (or nitrite), demonstrated that denitrification is carried out using as electron donor the internally stored PHA.

In order to increase denitrification and total nitrogen removal, after 239 days, DO concentration during Aeration 1 was reduced to 6  $\text{mg L}^{-1}$  and the phase was shortened to 60 minutes (Stage III). The presence of anoxic zones in the inner part of granules is a basic requirement for the occurrence of simultaneous nitrification and denitrification; the lower the dissolved oxygen concentration in the bulk liquid, the lower the oxygen penetration depth and consequently, the higher the anoxic volume inside the aggregate [14, 35]. According to Equation 8 and to the dissolved oxygen concentration during Stage II and Stage III ( $\text{DO}_{\text{II}}=9 \text{ mg L}^{-1}$ ;  $\text{DO}_{\text{III}}=6 \text{ mg L}^{-1}$ ), the estimated oxygen penetration depth ( $\delta_{\text{pf}}$ ) decreased from approximately 0.74 to 0.60 mm, after DO concentration was decreased from 9 to 6  $\text{mg L}^{-1}$ . As result of the increased anoxic volume, total nitrogen removal reached up to 90%.

The typical patterns of COD, phosphate and nitrogen concentrations during a cycle are shown in Figure 4.21.

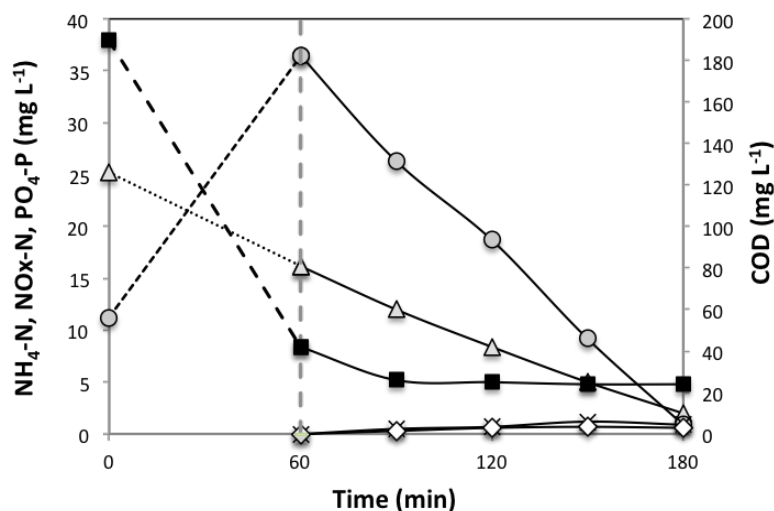


Figure 4-21. Concentration patterns of COD (■), NH<sub>4</sub>-N (▲), NO<sub>x</sub>-N (×), NO<sub>3</sub>-N (◇) and PO<sub>4</sub>-P (●), during a cycle.

The difference between the ammonium concentration expected at the end of the feeding phase, based on the influent concentration and on the dilution in the reactor after exchanging 50% of the volume, and the concentration experimentally measured is due to the anaerobic ammonium adsorption to granules and/or ammonium containing minerals (e.g. struvite) precipitation [36, 37].

Ammonium adsorption has been observed in activated sludge [38], biofilms [39, 40] and aerobic granular sludge [36] and results from an ion exchange process associated with the presence of the EPS matrix and the precipitates formed on these polymers, which act like ion exchangers [28]. In aerobic granular systems, P-release during the anaerobic phase promotes the precipitation of phosphates into diverse minerals [41], among which struvite (magnesium ammonium phosphate) and K-struvite (magnesium potassium phosphate) have been frequently detected on granules surface [37, 42, 43]. Lin et al. [37] demonstrated that the presence of K-struvite enhances ammonium adsorption during anaerobic feeding, acting like a potassium source for ion exchange with ammonium. Bassin et al. [36] showed the reversibility of this process (> 90% of the ammonium adsorbed can be desorbed during the aerobic phase and be available for nitrification) and used a Langmuir isotherm to describe the adsorption process. The adsorption value measured in this study for an ammonium influent concentration of 20 mg L<sup>-1</sup> (0.9 mg NH<sub>4</sub>-N g TSS<sup>-1</sup>) is in accordance to that expected from the adsorption isotherm (approximately 1 mg NH<sub>4</sub>-N g TSS<sup>-1</sup>) reported by Bassin et al. [36].

In conclusion, results showed that simultaneous COD, nitrogen and phosphorous removal was achieved and removal efficiencies registered at the end of the experiment are reported in Table 4.6.

**Table. 4.6. Average steady-state removal efficiencies.**

Parameters	Units	R1
NH <sub>4</sub> -N removal efficiency	(%)	95
TN removal efficiency	(%)	90
P removal efficiency	(%)	96
COD removal efficiency	(%)	95

#### 4.1.6 Conclusions

Aerobic granulation of activated sludge was successfully achieved in a laboratory-scale SBR with no pH and temperature control, fed in plug-flow conditions with a combination of synthetic medium and a domestic sewage, with the domestic sewage accounting for the 94.6% v/v of the combined influent. Mature granules showed a smooth outer surface, a mean diameter of roughly 1.5 mm and SVI<sub>30</sub> values below 20 mL gTSS<sup>-1</sup>.

The start-up of the reactor at low oxygen concentration (DO concentration set between 4 and 3.9 mg L<sup>-1</sup>) led to inadequate anaerobic COD storage and carbon removal carried out by conventional heterotrophs instead of slow growing organisms. Acetate uptake by PAO is highly dependent on DO concentration and therefore the start-up of a new system should rather take place at high DO concentration, which enhances poly-P storage and consequentially increase the anaerobic COD storage. This operating condition resulted essential to achieve a good PAO enrichment in the system and an actual feast-famine regime, both essential requirements for granules long-term stability. In fact, since granules stability is dependent on the growth rate of the microorganisms, selecting for slow growing bacteria is crucial to improve the stability of the process and nutrient removal performances. Results suggest that only after achieving steady stable PAO population, DO concentration can be decreased, in order to enhance total nitrogen removal efficiency and decrease the aeration costs.

Regarding reactor removal performances, simultaneous COD, nitrogen and phosphorous removal was achieved. C-removal was performed anaerobically by slow growing organisms (a fraction of which was also responsible for denitrification process), leading to a compact and stable structure of the granules. Low nitrification efficiency of the early stages, highlights the

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importance of the solid retention time; initially the growth of nitrifying biomass on filamentous sludge, characterized by a short SRT, led to a washout of nitrifiers from the system, leading to high ammonium concentration in the effluent. On the contrary, when a complete granular system was achieved, the development of nitrifiers on granules resulted in their retention inside the reactor and to a progressive population enrichment, which remarkably increased  $\text{NH}_4\text{-N}$  removal.

Moreover, initial low nitrification efficiency confirms also how biological kinetics depend on temperature, with nitrifiers being the most sensitive. Therefore, special attention must be addressed to the start-up period of a reactor run with no temperature control, preferring warm periods of the year, so that the decrease in nitrification rate due to fall-winter lower temperature can be overcome by higher biomass concentration in the reactor.

In conclusion, the results of this laboratory-scale study shows that aerobic granular sludge is a promising technology for wastewater treatment. However running a granular SBR requires an accurate control in order to allow different biological processes to take place in the same reactor during one cycle. Besides that, the most favourable operating conditions identified for microorganisms could not be the most suitable in terms of costs and therefore they must be adjusted so to find the best compromise between effluent quality demands and operating costs due to aeration. Further research is then needed to investigate unknown aspects of the process, which can be summarized as follows:

- Evaluation of the time needed for the start-up of a Granular Sequencing Batch Reactor directly at oxygen saturation levels, with specific attention at monitoring the establishment of nitrifying and slow growing organisms populations;
- Assessment of granules removal performances and long-term stability at lower DO concentrations;
- Monitoring the dynamic of microbial population through molecular tool, as Real-Time PCR (qPCR), Fluorescent In-situ Hybridization (FISH) and Denaturing Gradient Gel Electrophoresis (DGGE).
- Investigation of the influence of other variables (e.g., shear force), which may affect oxygen penetration inside the granule and consequently may play a role in the establishment of a stable population of slow growing organisms.

Finally, all the knowledge obtained from the above-mentioned laboratory-scale studies will be applied for the scale up to a pilot plant and to achieve a stable *inoculum* for the subsequent inhibitory tests.



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# **CHAPTER 5**

## **INHIBITORY EFFECT OF VETERINARY ANTIBIOTICS ON AEROBIC GRANULES**

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## Outline

In this chapter, the inhibitory effect of selected veterinary antibiotics was investigated for two microbial populations present within aerobic granules and responsible for the nitrogen and phosphorus conversion bioprocesses. The toxicity of different concentrations of doxycycline, tiamulin and enrofloxacin was assessed for ammonium oxidizing bacteria (AOB) and phosphorus accumulating organisms (PAO) collected from an aerobic granular sequencing batch reactor (SBR). Biomass was spiked with fixed pharmaceutical concentration and, subsequently, batch activity tests were conducted under fully aerobic conditions to determine the specific ammonium uptake rate (sAUR) and the specific phosphorus uptake rate (sPUR). Results obtained showed none or little inhibition of the three veterinary antibiotics on ammonium and phosphorus removal efficiency, thus demonstrating a high resistance of aerobic granular biomass towards selected toxic compounds. Moreover, since a maximum of 30-50% inhibition of aerobic granules activities was detected exclusively for concentration values highly exceeding those expected for real swine wastewaters, it is reasonable to conclude that aerobic granular biomass could be successfully applied in the treatment of this kind of effluents, resulting an efficient alternative to activated sludge process.

## Introduction

Intensive swine production is to date one of the most important sources of polluted and nutrient-rich wastewaters, which may be treated before being discharged to receiving water bodies or used for land application. In a conventional activated sludge process nitrogen is removed through autotrophic nitrification and heterotrophic denitrification, while the enhanced biological phosphorus removal (EBPR) can be achieved through an enrichment of the PAO culture.

Nowadays, innovative biological processes (e.g. biofilms and granular sludge) can represent an advantageous alternative to conventional activated sludge process. In fact, the application of granular biomass can overcome universal problems of settling and solid-liquid separation, eliminate multi-redox potential tanks necessary in conventional biological WWTPs, and the presence of an extracellular matrix of EPS ensures a better resistance to toxic pollutants [1,2]. Moreover, nutrient removal can occur simultaneously within granular biomass because of autotrophic growth at the aerobic surface (AOB) and heterotrophic growth inside the granule (PAO). All granules are, in fact, characterized by a concentric multi-layered structure, where aerobic, anoxic, and anaerobic zones characterized by different redox potentials can be defined along the direction of oxygen transfer within the granule. The different redox

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potentials allow for simultaneous COD, nitrogen, and phosphorus removal by encouraging the growth of aerobic, facultative, and obligate anaerobic bacteria [3].

To date, the suitability of aerobic granules for the treatment of industrial wastewaters has been investigated for different matrix [4-10] and increasing attention is paid to the utilization of this technology for swine slurry treatment [11-13]. However, swine wastewaters contain high concentrations of veterinary antibiotics, which could exert an antimicrobial activity towards all these microbial populations, causing a reduction of the treatment capacity. To date, very little attention has been paid to the interaction of antibiotics and microbial populations present within aerobic granular sludge. Amorim et al. [14] investigated the inhibitory effect on nutrient removal efficiency and microbial dynamics of an aerobic granular SBR exposed to fluoroquinolones shock loadings. Results obtained revealed that organic removal, as well as nitrifying activity, was not affected, although denitrifying and PAO activity was reduced. Shi et al. [15, 16] investigated respectively the effect of the short- and long-term exposure to tetracycline on aerobic granular biomass and indicated that nitrogen utilization rates were decreased after 12-hour exposure to 20 mg L<sup>-1</sup> or after more than 20-day exposure to 10 mg L<sup>-1</sup>. As the available literature is limited to only few kinds of antibiotics, a deeper evaluation regarding the potential toxicity of these pharmaceuticals on granular biomass stability and nutrient removal processes is then required. Aim of this chapter is therefore the evaluation of the inhibitory effect of selected pharmaceuticals on the nitrogen and phosphorus removal processes taking place within aerobic granules. Specifically, the action of different concentrations of doxycycline (DOX), tiamulin (TIA) and enrofloxacin (ENR) was assessed on the specific ammonium and phosphorus uptake rate.

## Materials and methods

### Chemicals

The veterinary pharmaceutical products (doxycycline hyclate, tiamulin fumarate and enrofloxacin) were purchased (≥98% purity) from Sigma-Aldrich (St. Louis, MO, USA). Concentrations tested are reported in Table 5.1.

**Table. 5.1. Tested antibiotics concentrations.**

Antibiotic	Concentration (mg L <sup>-1</sup> )
Doxycycline	100
Tiamulin	500
Enrofloxacin	200

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These concentrations exceed the expected values for influent swine wastewaters [17-22], but they were chosen taking into account the possibility of exceptional, temporary and massive antibiotics administration, which may lead to concentration peaks, and hence in order to exclude any toxic effect of the target compounds on aerobic granular sludge.

#### *Granular sludge and reactor operation*

Aerobic granules were cultivated in a laboratory-scale SBR, with an internal diameter of 8 cm, total working volume of 3 litres, volume exchange ratio of 50%, fed with a mixture of synthetic medium and domestic sewage from a municipal WWTP (San Colombano, Florence, Italy). Conventional activated sludge, taken from the same WWTP, was used as inoculum. The reactor was operating sequentially in 3-hour cycle comprising 60 minutes of anaerobic feeding, 60 minutes of Aeration 1 (with DO concentration set between 6 and 5.9 mg L<sup>-1</sup>), 50 minutes of Aeration 2 (with DO concentration set between 2 and 1.5 mg L<sup>-1</sup>), 2 minutes settling and 3 minutes discharging. DO concentration inside the reactor was regulated through a DO sensor connected to a control system. Fine bubbles supplied by a porous stone, placed at the bottom of the reactor, were used to increase DO concentration to the higher set point limit, while decreasing the oxygen saturation at lower levels occurred by means of coarse bubbles introduced at the bottom of the reactor. A set of two peristaltic pumps was used to introduce in plug-flow conditions the concentrated feeding solution and the domestic sewage through the settled sludge bed. The composition of the synthetic medium was the following: 8.6 g L<sup>-1</sup> NaAc, 2.38 g L<sup>-1</sup> (NH<sub>4</sub>)HCO<sub>3</sub>, 1.965 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>. Each cycle 72 mL of the synthetic medium was added to the reactor together with 1500 mL of domestic influent, in order to achieve influent concentrations of 320 mg COD L<sup>-1</sup>, 20 mg NH<sub>4</sub>-N L<sup>-1</sup>, and 20 mg PO<sub>4</sub>-P L<sup>-1</sup>, while total influent COD, NH<sub>4</sub>-N and PO<sub>4</sub>-P concentration were higher due to the COD, NH<sub>4</sub>-N and PO<sub>4</sub>-P contained in the domestic sewage. The reactor was running with no pH and temperature control; the SRT was around 70 days and the HRT was 0.25 days. For a detailed description of the system, see Chapter 4 (Section 4.3.1).

#### *Short-term inhibition tests aerobic granules*

Batch tests were performed under continuous aeration (DO > 6 mg L<sup>-1</sup>) at 20°C. The inhibitory effect of veterinary antibiotics was investigated on ammonium oxidizing bacteria (AOB) and phosphorus accumulating organisms (PAO), through the evaluation of the specific ammonium uptake rate (sAUR) and the specific phosphorus uptake rate (sPUR).



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To monitor nitrification process, granules were taken from the reactor at the end of the operational cycle and were aerated for 1 hour, in order to deplete residual ammonium concentration. To monitor phosphorus uptake rate, granules were taken from the reactor at the end of the anaerobic feeding phase, when bacteria have anaerobically stored all the available acetate. Granules were then washed with tap water and sieved, so to eliminate all cell fragments or suspended sludge. Subsequently the same amount of biomass (in terms of gWW) was distributed in 320 mL bottles filled with 200 mL of a synthetic solution containing: 88.7 mg L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 35.0 mg L<sup>-1</sup> KCl, 10 mL L<sup>-1</sup> trace element solution [23] and 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer to keep the pH constant at 8 (specific operating pH value during the cycle). A pulse of concentrated stock solution of the above-mentioned antibiotics was then added, and the bottles placed in a thermostatic shaker, at 20°C and 180 rpm, for 24-hour (Fig. 5.1).



**Figure 5-1. Experimental set-up.**

At the end of the exposure time, ammonium or phosphate were added so to achieve a non-limiting concentration (e.g., 25 mg L<sup>-1</sup>). 15 minutes after substrates addition, needed for metabolic pathways reactivation, a 2-hour test started; liquid samples were collected every hour and ammonium or phosphate concentrations measured. The hourly uptake rates of the exposed and unexposed biomass were calculated as average of the 2-hour rate measured during the entire test. Specific ammonium and phosphate uptake rate were then obtained by dividing the hourly uptake rate by the amount of VSS present inside the vial. To evaluate the inhibitory effect of veterinary antibiotics tested, the percentage of activity maintained after the exposure to different antibiotic concentrations was calculated with respect to the activity of the unexposed biomass (control). Each test was performed in quadruplicate.

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## Analytical methods

Ammonium ( $\text{NH}_4\text{-N}$ ) and phosphate ( $\text{PO}_4\text{-P}$ ) were determined spectrophotometrically using commercial test kits according to the protocol of the manufacturer (brand: Dr. Lange test kits, Hach-Lange GmbH, Düsseldorf, DE; kits LCK 303 for ammonium, LCK 348 for phosphate) and determined on a designated spectrophotometer (Dr. Lange 3900). All liquid samples were filtered at  $0.45\ \mu\text{m}$  before analysis. The concentrations of solids as Total Suspended Solids (TSS), and the fraction corresponding to the biomass as Volatile Suspended Solids (VSS), were determined according to the Standard Methods [24].

## Results and discussion

sAUR ( $\text{mg NH}_4\text{-N g}^{-1}\text{ VSS h}^{-1}$ ) and sPUR ( $\text{mg PO}_4\text{-P g}^{-1}\text{ VSS h}^{-1}$ ) were selected to assess the short-term effect of the exposure to DOX, TIA and ENR on granular populations of ammonium oxidizing bacteria and phosphorus accumulating organisms. The hourly sAUR and sPUR registered for the exposed biomass were compared to those obtained for the unexposed biomass and the average percentages of AOB and PAO activity maintained after 24-hour exposure to target compounds concentrations are shown in Figure 5.2 and 5.3, respectively. Each bar represents the average of four replicates.

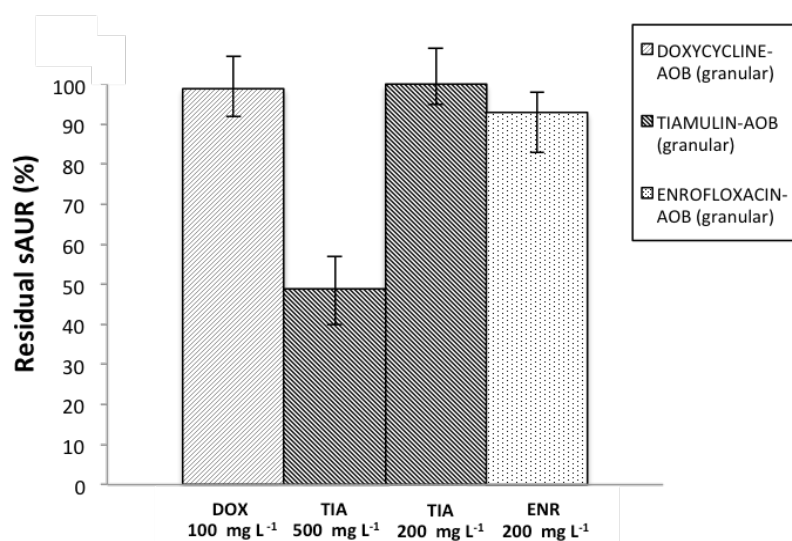


Figure 5-2. Average percentage of residual sAUR after 24h exposure to different antibiotics concentrations. Error bars represent the minimum and maximum value registered.

After 24-hour exposure to  $100\ \text{mg L}^{-1}$  DOX no inhibition in the nitrifying granular biomass activity was registered. On the contrary, a 50% inhibition of the sAUR was detected after the exposure to  $500\ \text{mg L}^{-1}$  TIA. In order to investigate more extensively the inhibitory effect of

TIA, another activity test was performed, reducing the antibiotic concentration to 200 mg L<sup>-1</sup>. At this concentration value no inhibition was detected. Regarding ENR, a negligible 7% inhibition occurred at concentration of 200 mg L<sup>-1</sup>. Shi et al. [15] investigated the short-term inhibitory effect of tetracycline on nitrifying granular sludge. After 12 hours exposure at 20 mg L<sup>-1</sup>, the respirometric activity of AOB and NOB resulted decreased by 15 and a 23% respectively, thus indicating that tetracycline induces stronger toxic responses than DOX. Moreover, results indicated NOB as the more susceptible bacteria, despite their inner placement inside the granule [25]. The authors stated that for NOB the toxic effect of tetracycline was much higher than the sheltering effect of the granule itself, concluding that the same antibiotic could affect diversely different microbial populations. A negligible 7% reduction of the NH<sub>4</sub>-N utilization rate caused by concentrations of 200 mg ENR L<sup>-1</sup> is in accordance with the results achieved by Amorim et al. [14], who investigated the removal performances of an aerobic granular SBR exposed to fluoroquinolones shock loadings. Results obtained revealed that nitrifying activity was not affected neither by single cycle shock of ofloxacin, norfloxacin and ciprofloxacin at concentrations up to 32 µM, nor when fluoroquinolones were continuously present in the inlet feeding during 11 consecutive days. Although the activity of AOB was not inhibited, a decline on both the anaerobic P-release and the aerobic P-uptake was observed. The higher susceptibility of phosphate accumulating organisms to fluoroquinolones is in accordance to data shown in Figure 5.3, where the average percentages of activity maintained by PAO after 24-hour exposure to the three selected pharmaceuticals are disclosed.

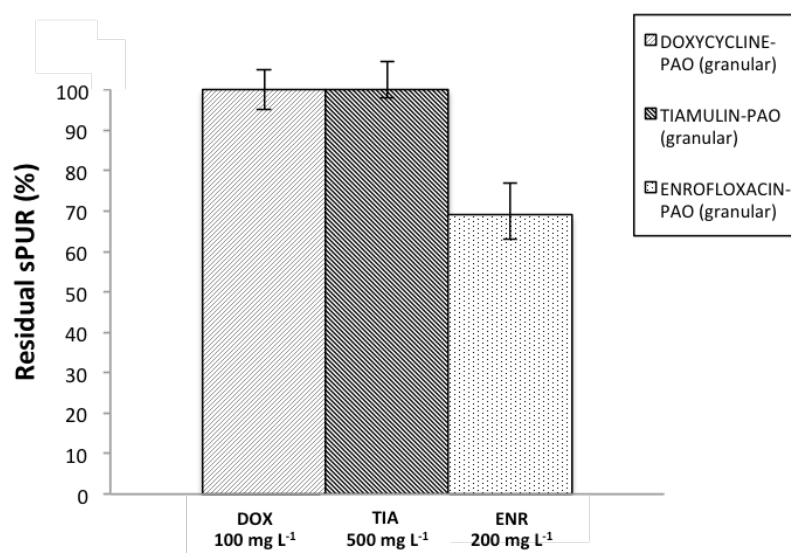


Figure 5-3. Average percentage of residual sPUR after 24h exposure to different antibiotics concentrations. Error bars represent the minimum and maximum value registered.

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In fact, while no inhibition was detected after 24-hour exposure to either 100 mg L<sup>-1</sup> DOX or 500 mg L<sup>-1</sup> TIA, sPUR of biomass exposed to 200 mg L<sup>-1</sup> ENR was reduced by 30%. Data thus confirm the results obtained by Amorim et al. [14] that fluoroquinolones exert a stronger inhibitory effect on phosphate accumulating organisms rather than ammonium and nitrite oxidizing bacteria.

Moreover, these data confirm also what was postulated by Shi et al. [15] regarding the different inhibition that a certain compound can cause on different microbial species, despite their placement inside the granule. In fact, in this specific case, PAO were more affected than AOB, although they are generally distributed in deeper layers. Specifically, granules are characterized by a concentric multi-layer structure; aerobic, anoxic and anaerobic zones can be detected along the direction of mass transfer, providing appropriate ecological micro-niches for aerobic, facultative and obligate anaerobic bacteria [26]. Therefore the typical structure of a granule presents ammonia oxidizing bacteria dominated within the first 200 µm below the granule surface, nitrite-oxidizing bacteria in a deeper layer between 200 and 300 µm, followed by PAO, GAO and denitrifiers (DPAO and DGAO) in the up following anoxic zones [27]. However, it must point-out that the spatial distribution is not so clearly defined, especially for AOB, NOB and PAO-GAO, which all compete for oxygen in the outer layer.

Data obtained display the high resistance of aerobic granular sludge to high concentrations of target compounds, thus demonstrating that a sheltering effect due to the specific granular architecture occurs. Moreover, the presence of an extracellular matrix of EPS enhances this protection and ensures a better resistance to toxic compounds [1, 2]. Under standard culture conditions the EPS production is normally low; an increase in this production occurs when bacterial cells are exposed to external stresses. These stresses can be divided into two main groups [28]: environmental changes which can alter the microbial community, increasing or decreasing the amount of EPS-producing bacteria, and environmental changes which can modify the metabolic pathway of EPS production of the unchanged microbial community. In the presence of toxic substances, microbial cells produce more EPS to protect themselves from the harsh environment [16, 29-31]. Moreover, EPS matrix secreted by aerobic granules exhibit a higher density than a normal exopolymeric matrix and behave as a strong gel across the normal operating pH range of a wastewater treatment plant (between 6 and 8.5) [32], thus providing a higher sheltering effect towards the underlying biomass.

Typically, antibiotic concentrations in manure vary in a range between 1 to 10 mg L<sup>-1</sup>, but occasionally they may reach levels of 200 mg L<sup>-1</sup> [33]. According to target pharmaceuticals concentrations measured in swine wastewaters [17-22], it is reasonable to state that the short-term exposure to these antibiotics won't affect AOB and PAO microbial activities, since a

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maximum of 30-50% inhibition was only registered at concentrations highly exceeding the foreseen values. Nevertheless, in order to assess the real susceptibility of granular sludge to veterinary antibiotics and then to pharmaceutical-rich wastewaters, the effect of the long-term exposure must be evaluated, and the assessment of the inhibitory effect caused by the simultaneous administration of different antibiotics must be considered.

## **Conclusions**

The short-term inhibitory effect of three veterinary antibiotics commonly administered to Italian swine was evaluated for granular populations of AOB and PAO, in order to achieve an overall perspective regarding the inhibitory effect of selected pharmaceuticals on the via nitrite nitrogen and phosphorus removal processes. Results obtained in presence of high concentrations of DOX, TIA and ENR showed none or little inhibition in the specific microbial activities. According to target antibiotics concentrations expected for swine wastewaters, it is reasonable to state that granular AOB and PAO activity is not affected by 24-hour exposure to those chemicals. AOB and PAO specific uptake rates were, in fact, reduced at most by 50 and 30% respectively, only in presence of TIA and ENR concentrations highly exceeding the foreseen values.

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# **CHAPTER 6**

## **COMPARISON BETWEEN VETERINARY ANTIBIOTICS EFFECT ON GRANULAR AND SUSPENDED BIOMASS**

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## Outline

In this chapter, the inhibitory effect of veterinary antibiotics was assessed for the nitrogen conversion bioprocesses commonly applied for wastewater treatment by means of both granular and suspended biomass. The toxicity of doxycycline, tiamulin and enrofloxacin was evaluated for ammonium oxidizing bacteria and denitrifiers developed as flocculent aggregates, through the measurement of the specific ammonium uptake rate (sAUR) and specific nitrate uptake rate (sNUR), respectively. Monitoring of nitrification and denitrification processes was achieved by means of short-term batch experiments conducted respectively under aerobic and anoxic conditions. The toxicity exerted by target compounds on the abovementioned populations was then compared to that measured in Chapter 3 and Chapter 5 for granular guilds with the same functionality: granular ammonium oxidizers and anammox bacteria, respectively. Results highlighted the higher resistance of the microbial guilds performing aerobic ammonium oxidation present in granules towards all the tested antibiotics compared to AOB populations present in conventional activated sludge flocs. Anammox bacteria susceptibility was instead depending on the specific antibiotic tested, leading to an overall inhibition nearly comparable to that of conventional flocculent denitrifiers.

## Introduction

Modern livestock production is mainly based on intensively raised indoor systems, leading to the production of pharmaceuticals and nutrient-rich wastewaters, which need to be treated before being discharged to receiving water bodies. Livestock manure represents one of the most significant sources of nitrogen, whose larger fraction is present in the ammonium form. Moreover, swine wastewaters contain high concentrations of veterinary antibiotics prescribed to animals in order to prevent animal infections, cure diseases and as growth promoters. Since antibiotics are just barely metabolized by the animals [1], the excreted parent compounds, as well as their metabolites, may enter WWTPs through raw manure. Conventionally nitrogen removal from swine wastewaters can be biologically achieved through autotrophic nitrification and heterotrophic denitrification. The biological oxidation of ammonia is the primary step in the nitrification process and the activity of ammonium oxidizing bacteria (AOB) was demonstrated to be negatively affected by the presence of antimicrobial agents [2-4]. Nowadays, innovative biological processes (e.g., biofilms and granular sludge) have been regarded with increasing interest according to their several advantages over conventional activated sludge in terms of lower operational costs and space requirements, capacity to withstand high loading rates [5] and higher resistance of biofilm-embedded cell to toxic

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compounds compared to planktonic cells [6,7]. To date, the inhibitory effect of different veterinary pharmaceuticals was studied for different types of biomass involved in the nitrogen removal via nitrite process [8], but there is a lack of information regarding the inhibitory effect of veterinary antibiotics on aerobic granular sludge. Moreover, a comparison between the susceptibility of the microbial populations performing aerobic ammonium oxidation present within granules and conventional activated flocs towards these compounds has never been documented. Hence, aim of this study was the comparison between the inhibitory effect exerted by doxycycline (DOX), tiamulin (TIA) and enrofloxacin (ENR) on granular and flocculent ammonium oxidizing bacteria cultivated on the same domestic influent. In fact, since biological treatment process creates a suitable environment for microbial resistance development [9], the effect of target antibiotics was assessed for microbial population grown on the same domestic influent, which presumably evolved similar adaptation abilities.

Regarding the second step of nitrogen removal, the reduction of nitrate and/or nitrite to dinitrogen gas, can be biologically achieved through a conventional heterotrophic denitrification process, or through a completely autotrophic nitrogen removal process, which involves nitrification and anaerobic ammonium oxidation (anammox). Partial nitrification/anammox process has been extensively studied because of its potential engineering application related to the reduction of operational costs. Furthermore, recent studies demonstrated its suitability for nitrogen removal from digested swine manure [10]. However, it has been demonstrated how several substances can inhibit the anammox biomass, thus reducing its advantages and future application [11]. The effects of several antibiotics were also studied on conventional denitrification process [12], indicating how denitrification rates are inhibited at environmentally relevant doses of the target compounds. Nevertheless, the comparison between the action of veterinary antibiotics on autotrophic (anammox) and heterotrophic denitrifiers has received little attention and was investigated for the first time by Alvarino et al. [8]. In the referred study, the impact of DOX was evaluated on the kinetics involved in the biogeochemical processes of nitrogen removal. Thus, after the evaluation of the respective inhibition grade of granular and flocculent AOB populations, the toxicity of target compounds was also assessed for heterotrophic and autotrophic bacteria performing the same denitrifying functionality and cultured from WWTPs treating domestic effluents, so to achieve an overall perspective regarding the specific suitability of different biomasses applied for nitrogen removal from antibiotic-rich swine wastewaters.

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## Materials and methods

### *Inoculum*

Flocculent activated sludge originated from San Colombano WWTP, Florence, Italy. The primary characteristics of the sludge used, such as the concentration of total suspended solid (TSS), and volatile suspended solids (MLVSS) were 7.5 gTSS L<sup>-1</sup> and 4 gVSS L<sup>-1</sup>, respectively.

### *Chemicals*

The veterinary pharmaceutical products (doxycycline hyclate, tiamulin fumarate and enrofloxacin) were purchased ( $\geq 98\%$  purity) from Sigma-Aldrich (St. Louis, MO, USA). Concentrations tested are the same previously tested for granular biomasses and are shown in Table 6.1.

**Table. 6.1. Tested antibiotics concentrations.**

Antibiotic	Concentration (mg L <sup>-1</sup> )
Doxycycline	100
Tiamulin	200-500
Enrofloxacin	200

### *Short-term inhibition tests on flocculent ammonium oxidizing bacteria*

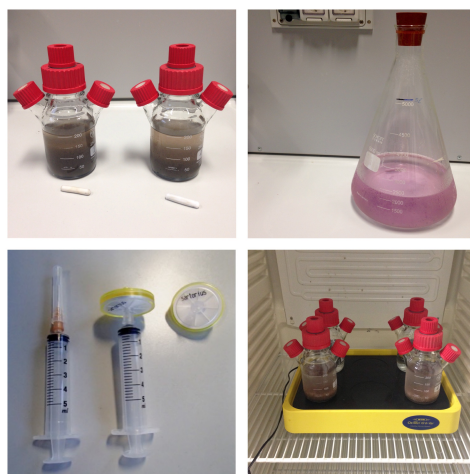
The specific ammonium uptake rate of conventional nitrifying activated sludge was measured by means of batch tests performed under fully aerobic conditions (DO > 6 mg L<sup>-1</sup>) at 20°C. Activated sludge was aerated for 1 hour, in order to deplete residual ammonium concentration. Consequently, the same amount of sludge (in terms of mL) was distributed in 320 mL bottles filled with 200 mL of a synthetic solutions containing: 88.7 mg L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 35.0 mg L<sup>-1</sup> KCl, 10 mL L<sup>-1</sup> trace element solution [13] and 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer to keep the pH constant at 7 (specific operating pH value of the reaction tank of the WWTP). A pulse of concentrated stock solution of the target compounds was then added, and the bottles were placed in a thermostatic chamber at 20°C and kept stirred by a magnetic mixer. After 24-hour exposure, biomass was spiked with a concentrated ammonium solution, in order to achieve an initial non-limiting concentration, and 15 minutes after substrate injection, needed for metabolic pathways reactivation, a 2-hour test started and nitrate concentration during time was recorded. The hourly uptake rate was calculated as average of the 2-hour rate, while sAUR was calculated by dividing the hourly

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uptake rate by the amount of VSS present inside the vial. To evaluate the inhibitory effect of veterinary antibiotics tested, the percentage of activity maintained after the exposure to different antibiotic concentrations was calculated with respect to the activity of the unexposed biomass (control). Each test was performed in quadruplicate.

#### *Short-term inhibition tests on flocculent denitrifiers*

The specific nitrate uptake rate (sNUR) of conventional heterotrophic denitrifiers developed within activated sludge was measured by means of batch tests performed under anoxic conditions, at 20°C. The same amount of sludge (in term of mL) was distributed in glass vessel (320 mL) filled with 200 mL of a synthetic solution containing: 88.7 mg L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 35.0 mg L<sup>-1</sup> KCl, 10 mL L<sup>-1</sup> trace element solution [13] and 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer to keep the pH constant at 7 (specific operating pH value of the anoxic tank of the WWTP). Each vessel was provided with two lateral holes sealed with a puncturable rubber septum for both substrate injections and sampling. The liquid phase and the headspace were flushed with dinitrogen gas through porous stones and needles respectively, in order to guarantee anoxic conditions inside the reactor. A pulse of concentrated stock solution of the antibiotics tested was then added, and the bottles were placed in a thermostatic chamber at 20°C and kept stirred by a magnetic mixer (Fig. 6.1).



**Figure 6-1. Experimental set-up.**

At the end of the exposure time, nitrate and sodium acetate (as carbonaceous source) were added so to achieve a non-limiting concentration. The hourly uptake rate, sNUR and the percentage of activity maintained after the exposure to tested antibiotics were then calculated as described in the above section. Each test was performed in quadruplicate.

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## Analytical methods

Nitrate ( $\text{NO}_3\text{-N}$ ) and ammonium ( $\text{NH}_4\text{-N}$ ) concentrations were determined spectrophotometrically using commercial test kits according to the protocol of the manufacturer (brand: Dr. Lange test kits, Hach-Lange GmbH, Düsseldorf, DE; kits LCK 339 for nitrate, LCK 303 for ammonium) and determined on a designated spectrophotometer (Dr. Lange 3900). All liquid samples were filtered at  $0.45\ \mu\text{m}$  before analysis. The concentrations of solids as Total Suspended Solids (TSS), and the fraction corresponding to the biomass as Volatile Suspended Solids (VSS), were determined according to the Standard Methods [14].

## Results and discussion

### *Short-term effect of doxycycline, tiamulin and enrofloxacin on floccular biomass*

The toxicity of DOX, TIA and ENR was assessed for ammonium oxidizing bacteria and heterotrophic denitrifiers developed within floccular aggregates, through the measurement of the sAUR ( $\text{mg NH}_4\text{-N g}^{-1}\text{ VSS h}^{-1}$ ) and sNUR ( $\text{mg NO}_3\text{-N g}^{-1}\text{ VSS h}^{-1}$ ). Inhibition due to the exposure to the target compounds was assessed by comparing the average hourly sAUR and sNUR of the exposed biomass with that obtained for the unexposed biomass, and the percentages of microbial activity maintained after 24-hour exposure to different antibiotics concentrations are shown in Figure 6.2 and 6.3, respectively. Each bar represents the average of four replicates.

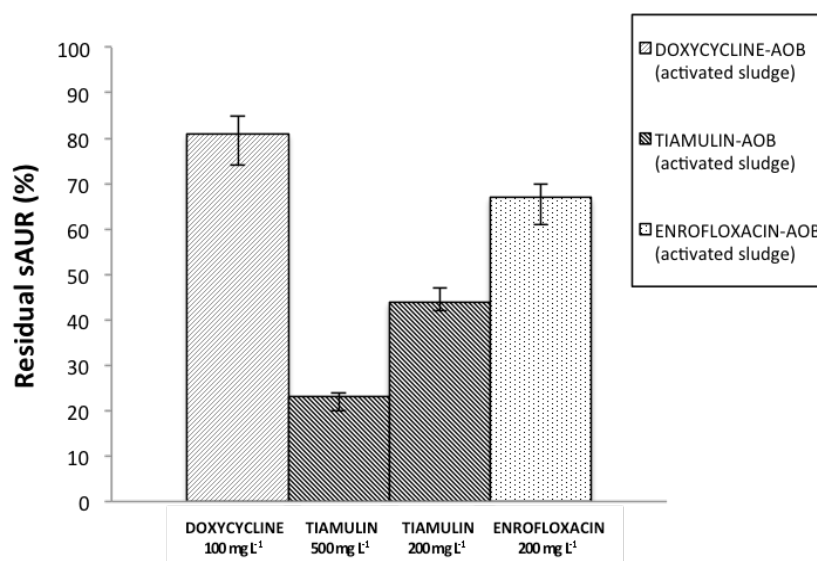
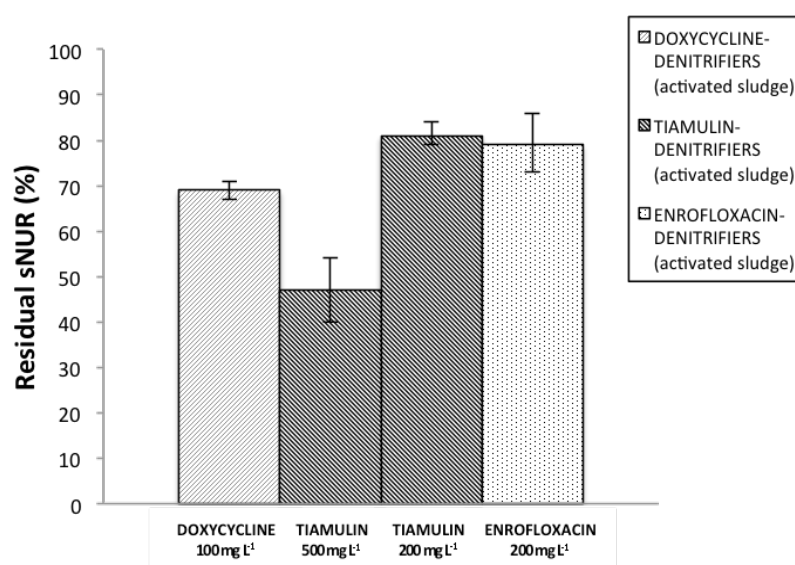


Figure 6-2. Average percentage of residual sAUR after 24h exposure to different antibiotics concentrations. Error bars represent the minimum and maximum value registered.

Figure 6.2 discloses the percentage of activity maintained by AOB population after 24-hour exposure to the target compounds. According to the experimental data, this microbial population discloses a significant susceptibility for each antibiotic tested. Specifically, sAUR decreased by 19% at DOX concentration of 100 mg L<sup>-1</sup>, by 77 and 56% at TIA concentration of 500 and 200 mg L<sup>-1</sup> respectively, and by 33% at ENR concentration of 200 mg L<sup>-1</sup>. Short-term tests on denitrifying bacteria (Fig. 6.3) show the occurrence of an inhibition equal or higher than 19% for all the target compounds. Specifically, sNUR was reduced by 31% at DOX concentrations of 100 mg L<sup>-1</sup>, by 53 and 19% at TIA concentrations of respectively 500 and 200 mg L<sup>-1</sup>, and by 21% at ENR concentrations of 200 mg L<sup>-1</sup>.



**Figure 6-3. Average percentage of residual sNUR after 24h exposure to different antibiotics concentrations. Error bars represent the minimum and maximum value registered.**

From a comparison between the inhibitory effect of the target compounds on nitrifying and denitrifying activities, AOB show a higher susceptibility to TIA and ENR, while conventional denitrifiers exhibit a higher sensitivity to DOX. Additionally, from the comparison of the inhibition induced by the same concentration of TIA and ENR on nitrifying and denitrifying biomass, TIA was the most toxic pharmaceutical for AOB, while denitrifiers show nearly the same susceptibility for both antibiotics. The toxicity of different concentrations of DOX on nitrification and denitrification process was also investigated by Alvarino et al. [8]. In the referred study sAUR was reduced respectively by 65 and 78% at DOX concentration of 50 and 250 mg L<sup>-1</sup>, while denitrifying activity of biomass acclimatized to high strength nitrogenous effluents showed a 19 and 25% inhibition of the nitrite uptake rate. DOX action on nitrifying and denitrifying bacteria acclimatized to low strength nitrogenous effluents was instead lower,

leading to sAUR reduction of 7 and 15.5%, and to a nitrite uptake rate reduction of 2 and 8% at concentrations of 100 and 250 mg L<sup>-1</sup>. Halling-Sørensen [2] assessed the toxicity of different antibacterial agents on the nitrification rate of conventional activated sludge and found that the presence of TIA, tetracycline, chlortetracycline and oxytetracycline inhibited this process. Fluoroquinolones have been reported to reduce the performance of the partial nitrification process [4, 15]. The influence of different concentrations of ciprofloxacin on this biological process was investigated by Gonzalez-Martinez et al., 2014 and results suggest that flocculent AOB are susceptible to the long-term exposure of low ciprofloxacin concentrations (70% reduction of ammonium oxidation at concentrations up of 350 ng L<sup>-1</sup>), suggesting that fluoroquinolones toxicity may increase with the exposure time.

#### *Comparison between veterinary antibiotics effect on granular and suspended biomass*

To determine which biological process applied for nitrogen removal is more stable towards veterinary antibiotics, the toxicity of selected target compounds was assessed for four microbial populations applied for nitrogen removal from the main and side-stream treatment: AOB developed within aerobic granules, AOB present in conventional activated sludge flocs, granular autotrophic denitrifiers (anammox) and heterotrophic denitrifiers developed within activated sludge flocs. Aim of this study is, in fact, the evaluation of the susceptibility grade of granular biomasses to high levels of toxic compounds, so to achieve a preliminary perspective regarding their possible application for the treatment of polluted swine wastewaters. In this scenario, identifying the higher resistance of granular aggregates compared to conventional activated sludge flocs, could be an additional advantage besides those already listed in the related Chapters. Results of the inhibition tests performed on AOB developed within aerobic granules and granular autotrophic denitrifiers (anammox) are described in Chapter 3 and 5, respectively. The comparison between the inhibitory effect of target antibiotics on granular and flocculent biomass is shown in Table 6.2.

**Table. 6.2. . Inhibition of microbial activities detected for granular and flocculent biomasses. ND=non detected.**

	DOX (100 mg L <sup>-1</sup> )	TIA (200 mg L <sup>-1</sup> )	(500 mg L <sup>-1</sup> )	ENR (200 mg L <sup>-1</sup> )
Granular AOB	ND	ND	51%	7%
Flocculent AOB	19%	56%	77%	33%
Granular denitrifiers (anammox)	17%	16%	35%	55%
Flocculent denitrifiers	31%	19%	53%	21%



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Data presented indicate that flocculent AOB disclosed higher sensitivity for each antibiotic assessed than AOB growing within aerobic granules, while anammox bacteria are slightly more resistant to DOX and TIA than flocculent denitrifiers, but more sensitive to ENR.

Anammox bacteria and conventional heterotrophic denitrifiers belong to different Phyla and disclose different metabolic pathways; it is therefore reasonable to suppose that this difference could affect diversely their vulnerability to antibiotic exposure. However, although the inhibitory effect of target compounds on anammox and flocculent denitrifiers is nearly comparable, the overcapacity, distinctive of granular systems, may counterbalance the reduction of the anammox bacterial activity, allowing a suitable management of the influent nitrogen load also in presence of toxic compounds. The overcapacity of biofilm systems originates from the presence of steep concentration gradients along the biofilm thickness, resulting in large part of the biomass operating under stringent substrate limiting conditions. Furthermore, the good settling properties facilitate the biomass retention inside granular systems allowing reactor operation at high biomass concentrations [16, 17].

The overall higher resistance exhibited by granular AOB populations towards target compounds could be ascribed to differences in the characteristics and amount of EPS produced, or in the microbial composition of the aggregates tested. On the one hand, although the ability to secrete EPS is widespread among bacteria, the strong selective pressures applied to achieve granulation process, enhance the production of EPS in granules [18]. Furthermore, there are significant differences between the EPS produced by sludge flocs and aerobic granules, with the latter being more compact and acting as a real structural gel at the operating pH of wastewater treatment processes [19, 20]. The peculiar gel-forming characteristics of aerobic granular EPS were demonstrated to be due to their exopolysaccharides content [21] and may be responsible for the higher sheltering effect towards the underlying biomass. On the other hand, Winkler et al. [22] demonstrated how different operational conditions applied in aerobic granular and flocculent sludge technology select for different genera of AOB, with *Nitrosomonas* being the dominant genus in flocculent sludge, whereas in granular sludge, *Nitrosomonas* and *Nitrospira* are present in equal amounts.

Therefore, inhibitory tests on pure cultures are required, so to verify the real susceptibility of these two genera towards target antibiotics, and ascribe the higher resistance of granular AOB to the diverse bacterial community or to the sheltering effect provided by the granular architecture and the specific EPS secreted by aerobic granules. In conclusion, taking into account the lower susceptibility to veterinary antibiotics and the several cost-saving advantages originating from the application of different granular technologies for nitrogen

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removal from swine wastewaters compared to the most common applied treatment-schemes, granular sludge result a promising process to be applied to this kind of nutrient-rich polluted wastewaters.

## Conclusions

The inhibitory effect of veterinary antibiotics on granular biomasses and conventional activated sludge was compared. The toxicity of DOX, TIA and ENR was assessed for four microbial populations applied for the main and side-stream nitrogen removal: AOB developed within aerobic granules, AOB present in conventional activated sludge flocs, granular autotrophic denitrifiers (anammox) and heterotrophic denitrifiers developed within activated sludge flocs.

Results presented highlight the higher resistance of granular AOB populations towards all the tested antibiotics compared to conventional flocculent nitrifiers. Instead, anammox bacteria resulted slightly more resistant for just two of the three target compounds than denitrifying flocs. In granular sludge, most likely, the unique settling properties, the overcapacity typical of biofilm systems, and the high production of specific EPS, makes microorganisms more suitable for the treatment of antibiotic-rich wastewaters. Specifically, although the ability to secrete EPS has been detected also for activated sludge, the strong selective pressures applied to achieve granulation process, enhance the production of EPS in granules. Furthermore, there are also significant differences between the EPS produced by sludge flocs and granules, with the latter being more compact and disclosing gel-forming characteristics. Beside these clear results, is however necessary to investigate more extensively the susceptibility of pure nitrifying cultures towards selected compounds in order to ascribe the higher resistance of granular AOB to the sheltering effect provided by the granular architecture and the specific EPS secreted, instead of the specific genera of AOB present. Moreover the evaluation of the response of nitrifying and denitrifying microbial activities under long-term exposure to selected antibiotics is also required. In fact, so to assess the real feasibility of granular biomasses for the treatment of manure, the resistance observed towards the short-term effect of tested antibiotics is not sufficient to achieve a detailed picture of the possible inhibition caused by the prolonged exposure to one or more antibiotics. Regarding this last aspect, future attempts should focus on the assessment of the microbial response to the long-term exposure to the single and combined administration of antibiotics. The combined addition of different antibiotics could, in fact, strengthen the effect due to a single administration, resulting in inhibition values higher than that caused by the single product.

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Since swine wastewaters are a mixture of different veterinary antibiotics, the combined presence of pharmaceuticals must be taken into account and their inhibitory effect assessed in detail.

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# **CHAPTER 7**

## **EVALUATION AND OUTLOOK**

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## Aim of the research

The main objective of this thesis was the evaluation of the suitability of granular sludge technology as an economically feasible alternative to conventional activated sludge for the treatment of swine wastewaters. The research mainly focused on the evaluation of the most advantageous operating conditions to improve aerobic granules formation and long-term stability, in order to scale-up this biological process into a stable full-scale system, and on the assessment of the inhibitory effect of veterinary antibiotics commonly present in these kind of wastes on two specific granular consortia: the anammox biomass and the aerobic granules, in order to evaluate their suitability for the side and main-stream treatment of manure. The experimental campaign included different laboratory-scale studies: the start-up of an aerobic granular Sequencing Batch Reactor and inhibition tests for the estimation of the toxicity of three veterinary antibiotics respectively on anammox granules, aerobic granules and conventional activated sludge.

## Evaluation

### *Cultivation of aerobic granules*

Aerobic granulation of activated sludge was achieved in a Sequencing Batch Reactor specifically designed and operated as a selective device, fed with a mixture of synthetic medium and domestic sewage. Granulation process and reactor removal performances were studied with the aim of translating experimental observations and results into a feasible full-scale process and to achieve the *inoculum* for the subsequent inhibition tests. Simultaneous COD, nitrogen and phosphorous removal was obtained and the operating parameters, which were found to influence mostly granules stability and removal performances can be summarized as follows:

- Selection of slow-growing organisms: high enrichment of slow growing organisms (PAO/DPAO and GAO/DGAO) is advantageous to increase granule long-term stability and allows for simultaneous nitrification-denitrification and phosphorus removal (SNDPR) process, thus enhancing the removal efficiency of the system. One of the most essential requirements in order to obtain stable granules is decreasing the growth rate of microbial species developing within the aggregate, by promoting the conversion of readily biodegradable substrates into slowly biodegradable polymers. PAO and GAO efficiently carry out this conversion, leaving no available substrate for



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conventional fast growing heterotrophs during the aeration phase, thus becoming the dominant species and avoiding filamentous overgrowth.

- Feeding strategy: plug-flow feeding from the bottom of the reactor, through the settled sludge bed, enhances granulation by selecting for heavier aggregates, characterized by good settling properties;
- Dissolved oxygen concentration: DO concentration during the start-up of a new system is a critical factor which can affect PAO development by influencing poly-P storage; therefore the start-up should take place at high DO concentration (saturation), which can be reduced once achieved a stable PAO population, in order to enhance total nitrogen removal efficiency and decrease the aeration costs.
- Temperature: low temperatures influence biological kinetics, with nitrifiers being the most sensitive; special attention must be addressed to the start-up period of a reactor run with no temperature control, preferring warm periods, so that the decrease in nitrification rate due to fall-winter lower temperature can be overcome by higher biomass concentration in the reactor.

#### *Inhibitory effect of veterinary antibiotics on anammox biomass*

The suitability of the anammox process for the side-stream treatment of digested manure was assessed through the evaluation of the short- and long-term inhibitory effect of three veterinary antibiotics commonly administered to Italian swine. Experimental results achieved can be summarized as follows:

- The short-term toxicity of DOX and TIA increases with higher concentrations and longer exposure time; while the short-term inhibition of ENR increases with higher concentrations, remaining almost stable during time. Short-term toxicity of the target compounds varies in sequence of DOX > ENR > TIA;
- The long-term inhibitory effect of DOX was confirmed to be stronger than that of ENR, although the latter reaches its maximum toxicity shortly after the administration. Moreover, ENR exhibits concentration-dependent bacterial killing, suggesting that bacteria can adapt to low concentrations, as reported for other fluoroquinolones.
- A synergistic effect was detected for the combined use of DOX and ENR. In fact, the combined use of these two antibiotics caused a greater inhibition than the sum of the single antibiotics use.

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- According to inhibition values recorded in presence of the maximum antibiotics concentrations foreseen for digester liquor, it is reasonable to suppose that the antibiotics tested do not represent a real hazard for the application of the anammox process because at that concentration levels, just a negligible inhibition was registered;

#### *Inhibitory effect of veterinary antibiotics on aerobic granules*

The suitability of aerobic granules as a practicable and cost-effective alternative to conventional activated sludge for the treatment of swine wastewaters was assessed through the investigation of the short-term toxicity exerted by DOX, TIA and ENR on two microbial populations present within aerobic granules and responsible for the nitrogen and phosphorus conversion bioprocesses. Experimental results achieved can be summarized as follows:

- None or little short-term inhibition of the target compounds was registered for AOB and PAO populations developed within aerobic granules. A inhibition of AOB and PAO specific uptake rates was registered exclusively for TIA and ENR concentration values highly exceeding those expected for real swine wastewaters;
- According to target antibiotics concentrations expected for swine wastewaters, it is reasonable to state that granular AOB and PAO activity is not affected by 24-hour exposure to selected chemicals;
- The distinctive granular architecture and the gel-forming characteristics of EPS synthesized by microbial species developing within aerobic granules, likely provide a sheltering effect towards the underlying biomass and ensure a better resistance to toxic compounds.

#### *Comparison between veterinary antibiotics effect on granular and suspended biomass*

The inhibitory effect of veterinary antibiotics was assessed for the nitrogen conversion bioprocesses commonly applied for wastewater treatment by means of both granular and suspended biomass. The toxicity of DOX, TIA and ENR was evaluated for AOB developed within aerobic granules, AOB present in conventional activated sludge flocs, granular autotrophic denitrifiers (anammox) and heterotrophic denitrifiers developed within activated sludge flocs. Experimental results can be summarized as follows:

- Granular AOB disclose higher resistance for each antibiotic tested than flocculent AOB. Anammox bacteria susceptibility was instead depending on the specific antibiotic

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tested, leading to an overall inhibition nearly comparable to that of conventional flocculent denitrifiers. However, although the inhibitory effect of target compounds on anammox and flocculent denitrifiers is nearly comparable, the overcapacity distinctive of granular systems, may counterbalance the reduction of the anammox bacterial activity, allowing a suitable management of the influent nitrogen load also in presence of toxic compounds;

- The higher resistance of aerobic granular biomasses to veterinary antibiotics, may be due to the high production of gel-like EPS, which form a denser matrix than a normal exopolymeric matrix secreted by activated sludge, to differences in the microbial composition of the aggregates and finally to the distinctive spherical architecture of granules.
- Taking into account the lower susceptibility to veterinary antibiotics and the several cost-saving advantages originating from the application of different granular technologies for nitrogen removal from swine wastewaters compared to the most common applied treatment-schemes, granular sludge result a promising process to be applied to this kind of nutrient-rich polluted wastewaters;

## Outlook

Over the last decades, granulation has been regarded with increasing interest according to granules advantageous characteristics. Anammox bacteria are considered one of the most innovative and sustainable biological nitrogen removal alternatives to traditional nitrification-denitrification technology, while aerobic granular sludge represent a suitable alternative to conventional activated sludge as a system offering solution to the problems common to flocculant biomass and able to remove C, N and P simultaneously. The application of the anammox process for the treatment of digested manure has already been reported and also if this treatment scheme has not been applied yet in full scale, it appears suitable for both an environmental and an economic point of view. On the other hand, previous studies confirmed that aerobic granular sludge is extremely promising for the treatment of effluents containing toxic compounds and recent research demonstrated that aerobic granules could be cultivated using swine slurry, even though further optimization of the system is still needed.

Regarding aerobic granules cultivation, although factors affecting biogranulation process has been extensively studied, granules instability is still one of the major problem, which has not been fully understood. Despite the operating conditions whose influence on granules stability arose during this study, many others factors could affect granulation process. One of the most

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important aspects influencing granular microbial composition identified in this thesis is dissolved oxygen concentration. Since oxygen concentration inside the granule can be related not just to the oxygen concentration in the bulk liquid, further research should focus on the assessment of other aspects related with oxygen transfer. For instance, the occurrence of a boundary layer at the water-granule interface could reduce oxygen diffusion towards the inner layers of the granule, thus resulting in a DO limitation. In this scenario, the hydrodynamic shear force may play a role too; in fact an increase in the shear force could induce a higher turbulent flow, which might decrease the boundary layer, thus enhancing oxygen penetration depth. Particular attention must be addresses to the shear force source, avoiding mechanical mixing, which was demonstrated to be detrimental for the formation of stable granules and thus preferring an increase in the airflow.

As direct measurements of DO profiles inside aerobic granules cultivated under different aeration rates have not been reported, further investigation might enhance the knowledge concerning oxygen transfer inside aerobic granules and might solve the DO bottleneck. Moreover, another factor which has not been investigated in this study, but that may influence DO penetration and may be influenced by the hydrodynamic shear force is granule size. Besides a possible regulation of oxygen diffusion through the applied shear force, and the consequent decrease of the boundary layer, also granule size might be controllable by applying increased or decreased shear force. Finally, as aerobic granules are expected to be used for the treatment of variable domestic or industrial influents, the knowledge obtained from laboratory-scale experiments should be applied for the scale-up to a pilot plant, in order to monitor granules performances under real operating conditions.

Regarding the suitability of the anammox process for the treatment of digested manure, experimental results suggest that the inhibition values caused by the single administration of the maximum antibiotics concentrations foreseen for digester liquor could be considered negligible. However, the establishment of a synergistic effect after the combined addition of DOX and ENR suggests that veterinary antibiotics can interact, leading to higher inhibition values. Hence, the toxicity originating by the concurrent administration of two or more veterinary antibiotics must be investigated more in detail. Similarly, although aerobic granules demonstrated to be very resistant to veterinary antibiotics, further investigations regarding the combined use of different antibiotics are recommended. Moreover, so to assess the suitability of granular sludge for the treatment of this kind of wastes under real conditions, the influence of continuous and extended administration of antibiotics is required. This experimentation could also highlight the occurrence of natural or acquired microbial resistance, thus selecting for microorganisms genetically able to face antibiotic-rich

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wastewaters. Analytical methods (e.g., High Performance Liquid Chromatography, HPLC) could also be applied to improve our understanding regarding the fate of antibiotics during biological treatment processes, so to evaluate adsorption and degradation ability of both aerobic and anaerobic granular sludge.

Finally, even if the biological treatment of antibiotic-rich wastewaters by means of granular biomass would result possible all things considered, particular attention must be paid to the effect of antibiotics residues in the environment. In fact, once released into the environment through manure application and the discharge of effluents from WWTPs, antibiotics may affect both terrestrial and aquatic organisms. The side effects of antibiotics and their metabolites on non-target organisms include: phytotoxicity, bioaccumulation phenomena, which may raise potential human health concerns through food chain, adverse impact on the reproduction and early life stages of different aquatic organisms, inhibition of important ecosystem bacteria, and induction of bacterial resistance.

Therefore strategies aimed at lowering the introduction and ecological effects of veterinary antibiotics in the ecosystems are needed, as well as a defined monitoring programme of the usage of pharmaceuticals in livestock farming. Moreover, as the removal efficiency of antibiotics during wastewater treatment processes is generally inadequate, physical and chemical pre-treatment methods, such as advanced oxidation processes, photodegradation, photocatalysis and activated carbon adsorption must be further optimized in order to reduce pharmaceuticals and by-products concentrations in WWTPs effluents.

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